PASKIN: Target Protein Identification and Kinase Activity Regulation by Phospholipids

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zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Universität Zürich

von

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Zürich, 2007

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To my grandmother, Eva Pfeufer

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1. ABBREVIATIONS

Aer	aerotaxis sensor receptor
AhR	aryl hydrocarbon receptor
AMPK	AMP-activated kinase
ArcB	aerobic respiration control sensor protein
ARNT	aryl hydrocarbon receptor nuclear translocator
BMAL	brain and muscle ARNT-like protein
CAF20	Cap-associated factor 20
CCAMK	calcium/calmodulin-dependent protein kinase
CLOCK	circadian locomoter output cycles kaput protein
DAG	diacylglycerol
DOG	dioctanoylglycerol
DNA-PK$_{CS}$	DNA-dependent protein kinase catalytic subunit
eEF	eukaryotic translation elongation factor
eIF	eukaryotic translation initiation factor
EGF	epidermal growth factor
Fos	Finkel-Biskis-Jinkins (FBJ) murine osteosarcoma virus oncogene, transcription factor
GLUT	glucose transporter
Gsy2	glycogen synthase 2
GDP	guanosine diphosphate
GST	glutathione-S-transferase
GTP	guanosine trisphosphate
H2AX	H2A histone family, member X
HERG	human *eag*-related gene
HIF	hypoxia-inducible factor
Hsp70	heat-shock protein 70
IP$_3$	inositol trisphosphate
MBP	maltose-binding protein
MIN6	mouse insulinoma cell line
NifL	nitrogen fixation regulatory protein
NPAS	neuronal PAS protein
NMR	nuclear magnetic resonance

p53	53 kDa tumor suppressor protein
p300	300 kDa protein, histone acetyl transferase
PA	phosphatidic acid
PAS	PER/ARNT/SIM
PASKIN	PAS domain kinase
PC	phosphatidylcholine
PDX-1	pancreatic duodenal homeobox-1 transcription factor-1
PE	phosphatidylethanolamine
PER	*Drosophila* period clock protein
PI	phosphatidylinositol
PIP_2	phosphatidylinositol bisphosphate
PLC/D	phospholipase C/D
PKC	protein kinase C
PMA	phorbol 12-myristate 12-acetate
PS	phosphatidylserine
PYP	photoactive yellow protein
Raf-1	root abundant factor, v-raf-1 murine leukemia viral oncogene homolog
S	sphingomyelin
SAP	SAF-A/B, Acinus and PIAS motif
SIM	*Drosophila* single-minded protein
Sds22	protein phosphatase, regulatory subunit 7
Snf1	sucrose non-fermenting 1 protein kinase
Sp1	stimulator protein 1, transcriptional factor
Tif11	translation initiation factor 1A
Ugp1	UDP-glucose pyrophosphorylase
VEGF	vascular endothelial growth factor
vWA	von Willebrand factor A domain

2. ZUSAMMENFASSUNG

In der Zelle stellt die Phosphorylierung von Proteinen eine wichtige Form der posttranslationellen Modifikationen dar. Kürzlich haben wir eine neue Serin/Threonin Kinase in Säugetieren identifiziert, die wir PASKIN genannt haben. PASKIN besitzt zwei PAS Domänen mit hoher Ähnlichkeit zu dem bakteriellen Sauerstoffsensor FixL, der in verschiedenen *Rhizobium* Arten vorkommt. PAS Domänen agieren als Proteinsensoren, die fähig sind, externe Reize wie zum Beispiel Veränderungen der Lichtintensität, des Sauerstoffpartialdrucks, des Redoxpotentials, und elektrischen Spannung sowie die Konzentration verschiedener Liganden zu messen. Zusätzlich können PAS Proteine von Säugetieren als Kopplungsstelle für die Dimerisierung von Transkriptionsfaktoren dienen. Beispiele dafür findet man in der Antwort auf körperfremde Substanzen, in der Adaptierung auf Hypoxie und in der Tagesrhythmusregulierung. Neben den beiden PAS Domänen besitzt PASKIN als katalytische Domäne eine Serin/Threonin Kinasedomäne, die auf Sequenzebene den AMP Kinasen ähnelt. Es wird vermutet, dass PASKIN durch die Bindung eines Liganden aktiviert wird. Ähnlich wie bei anderen Proteinkinasen kann die Bindung eines Liganden an die PAS Domäne die Autophosphorylierung von PASKIN stimulieren, die dann wiederum zu einer gesteigerten Zielproteinphosphorylierung führen könnte. In der Hefe wurde gezeigt, dass PASKIN unter Stressbedingungen die Speicherung von Glykogen reduziert sowie die Proteintranslation vermindert. In Säugetierzellen phosphoryliert PASKIN die Glykogen-Synthase und scheint glucoseabhängig an der Insulinproduktion von β-Zellen im Pankreas beteiligt zu sein.

PASKIN ist am stärksten in der postmeiotischen Phase der Spermatogenese exprimiert. Von uns durch homologe Rekombination erzeugte PASKIN knock-out Mäuse weisen eine normale Entwicklung, Wachstum, Fortpflanzung sowie keine Beeinträchtigung der Fruchtbarkeit auf. Um die Funktion von PASKIN während der Spermatogenese zu untersuchen, wurde ein monoklonaler, gegen die PAS Domäne gerichteter Antikörper hergestellt. Zudem wurde ein Zwei-Hybrid Screening mit einer HeLa cDNA Bibiliothek in der Hefe durchgeführt, um mögliche Interaktionspartner von PASKIN zu identifizieren. Ein identifiziertes Target von PASKIN ist der eukaryotische Translations-Elongationsfaktor eEF1A1, welcher sowohl mit der PAS A als auch mit der Kinase Domäne von PASKIN *in vitro* und *in vivo* interagiert. Die Phosphorylierung von eEF1A1 durch PASKIN an Threonin 432 könnte auf eine Aktivierung von eEF1A1 hinweisen, die dann zu einer erhöhten translationellen Elongation führt. Tatsächlich sind beide Proteine im Mittelstück des Spermiumschwanzes lokalisiert, was

darauf hindeuten könnte, dass die eEF1A1 Phosphorylierung durch PASKIN die translationelle Elongation im Spermium reguliert. eEF1A1 wird auch durch die Proteinkinase PKCδ phosphoryliert, deren Aktivität durch ein Gemisch aus Phosphatidylserin und Diacylglycerol stimuliert wird. Da ein endogener Ligand für die PASKIN Aktivierung bisher nicht bekannt ist, haben wir die beiden Kinasen bezüglich ihrer Aktivierung durch Lipide untersucht. Zum ersten Mal konnten wir zeigen, dass Phospholipide im Gegensatz zum sekundären Botenstoff Diacylglycerol die Autophosphorylierung von PASKIN stimulieren.
Neu ist auch, dass die durch die Phospholipase D gebildete Phosphatidylsäure mit PASKIN *in vitro* interagiert. Die Tatsache, dass Phosphatidylsäure und nicht das von der Phospholipase C gebildete Diacylglycerol an PASKIN bindet, belegt Phosphatidylsäure als Minimaldomäne der Phospholipide für die PASKIN Aktivierung. Zusätzlich wird die Autophosphorylierung von PASKIN durch Phosphatidylsäure erhöht, gleichzeitig wird aber die eEF1A1 Targetphosphorylierung inhibiert.
Über die funktionelle Rolle von PASKIN während der Spermatogenese ist sehr wenig bekannt. Daher wurde ein weiteres Zwei-Hybrid Screening mit der Kinase Domäne als „Köder“ und einer humanen Hoden cDNA Bibliothek in der Hefe durchgeführt. Wir konnten das multifunktionelle Protein Ku70 als einen weiteren Interaktionspartner von PASKIN in der Hefe identifizieren. Des weiteren konnte diese Interaktion in einem GST Pull-down Experiment *in vitro* bestätigt werden. Außerdem konnte gezeigt werden, dass Ku70 nicht durch PASKIN phosphoryliert wird, auch nicht in der Anwesenheit von Phospholipiden oder dem sekundären Botenstoff Diacylglycerol. Die Identifizierung der beiden Interaktionspartner eEF1A1 und Ku70 wird in Zukunft dabei helfen, die physiologische Funktion von PASKIN besser zu verstehen.

3. SUMMARY

Protein phosphorylation is an important form of posttranslational modification involved in cellular signal transduction pathways. Recently, we and others identified a novel mammalian serine/threonine kinase that we named PASKIN. PASKIN contains two PAS domains which are highly related to the oxygen sensor protein FixL from *Rhizobium* species. PAS domains act as protein sensors that are involved in the perception of light intensity, oxygen partial pressure, redox potentials, voltage and concentrations of certain ligands. Additionally, mammalian PAS domains serve as dimerization interfaces of transcription factors involved in the xenobiotic response, adaptation to hypoxia and circadian rhythm generation. Besides the two PAS domains of PASKIN, the catalytic serine/threonine kinase domain is similar to the AMP kinases. PASKIN activation appears to be stimulated in a ligand-dependent manner. Ligand-binding to the PAS domain of PASKIN stimulates kinase activity, probably resulting in target-phosphorylation as is known from other protein kinases. It has been reported that under stress conditions PASKIN downstream-target activation decreases glycogen storage and protein translation in yeast. PASKIN phosphorylates mammalian glycogen synthase and seems to be involved in glucose-stimulated insulin production in pancreatic β-cells.

PASKIN is highly expressed during the postmeiotic stage of spermatogenesis. PASKIN knock-out mice generated by homologous recombination show normal development, growth, reproduction and fertility. To elucidate the function of PASKIN, we generated a mouse monoclonal antibody against the PAS domain of human PASKIN. Furthermore we performed a yeast two-hybrid screening of a HeLa cDNA library to identify novel interaction partners of human PASKIN. The eukaryotic translation elongation factor eEF1A1 was identified as target of PASKIN. eEF1A1 interacts with the PAS A and the kinase domains of PASKIN *in vitro* and *in vivo*. We could also demonstrate that eEF1A1 is phosphorylated at Thr 432, probably leading to increased translational elongation. Surprisingly, these two proteins showed a similar localization in the midpiece of the sperm tail, suggesting that phosphorylation of eEF1A1 by PASKIN regulates translational elongation in sperm cells. It is known that the protein kinase PKCδ also phosphorylates eEF1A1 and is maximally stimulated by a mixture of phosphatidylserine and diacylglycerol. Because an endogenous ligand for PASKIN activation was unknown so far, we compared these two kinases with regard to their activation by lipids. We could show that phospholipids but not the second messenger molecule diacylglycerol are stimulators for PASKIN activity. The finding that phosphatidic acid produced by phospholipase D but not diacylglyerol generated by phospholipase C interacts with PASKIN, suggests phosphatidic acid as minimal moiety for PASKIN stimulation. Phosphatidic acid enhances PASKIN autophosphorylation. Additionally, we could show that phosphatidic acid inhibits eEF1A1 target phosphorylation by PASKIN.

To obtain more insights into the functional role of PASKIN during spermatogenesis, a further yeast two-hybrid screening using the kinase domain of PASKIN as bait and a human testis cDNA library was performed. The multifunctional protein Ku70 was identified as interaction partner of PASKIN. This interaction could be confirmed *in vitro*, but no PASKIN phosphorylation of Ku70 took place in the presence of phospholipids or the second messenger molecule diacylglycerol. The identification of eEF1A1 and Ku70 as novel PASKIN interaction partners will help to elucidate the physiologic function of PASKIN.

4. INTRODUCTION

PASKIN – a novel serine/threonine kinase

The combination of protein kinases and protein phosphatases greatly influences regulatory processes in the cell. In 1959, it was reported for the first time that phosphorylation/ dephosphorylation controls the activity of a protein (glycogen phosphorylase). Over the years the role of phosphorylation in regulating protein function became more important [1,2]. Now, over 1000 protein kinases are known in vertebrates. The human kinome consists of 518 protein kinase genes, constituting about 1.7% of all human genes, and they have been classified into over 57 families [2]. By database searches using the PAS (PER/ARNT/SIM) sequence as bait [3], our and other labs identified a novel serine/threonine kinase belonging to the family of PAS proteins, named PASKIN [4] or PASK [5]. Based on the high similarity to the AMP kinases, PASKIN is assigned to the family of calcium/calmodulin-dependent kinases (Fig. 1).

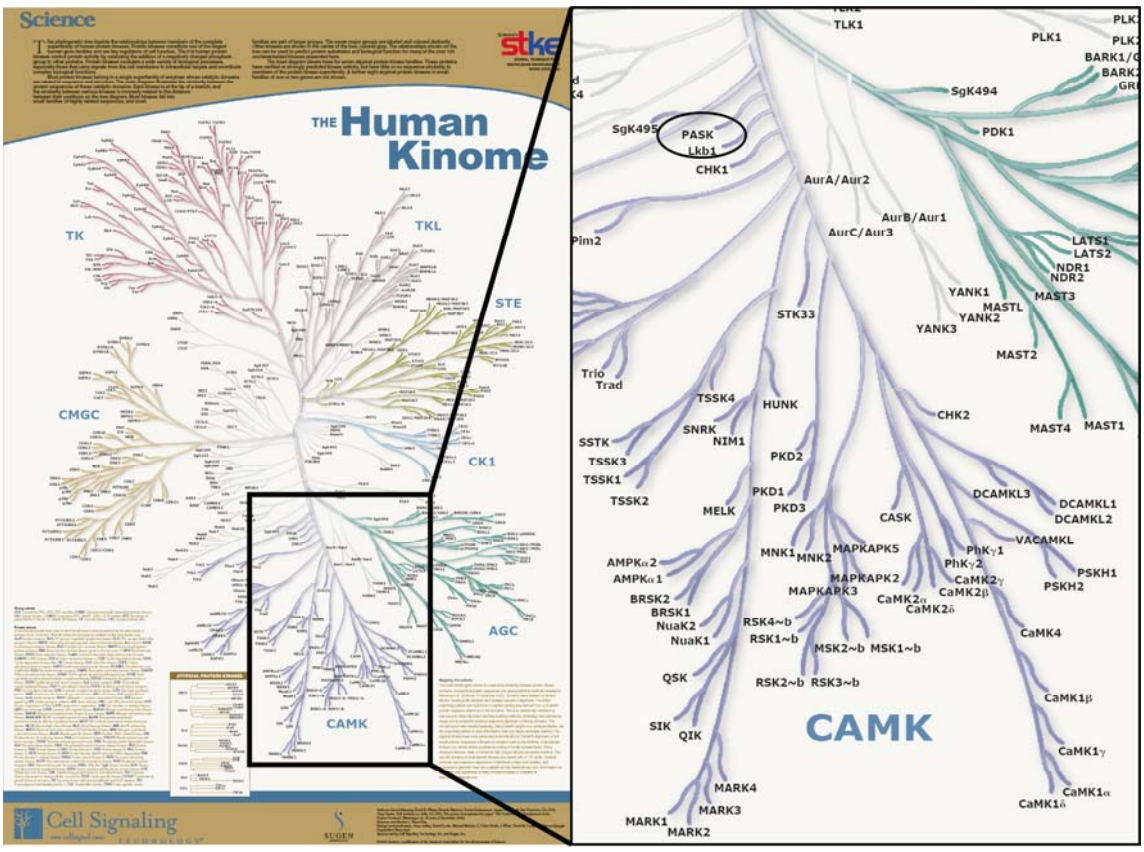

Figure 1: The human kinome. The phylogenetic tree depicts the relationships between members of the complete superfamily of human protein kinases (www.cellsignal.com). PASKIN or PASK is assigned to the family of calcium/calmodulin-dependent kinases.

The domain architecture of PASKIN resembles that of the oxygen sensor protein FixL from *Rhizobium* species which contains a heme-bearing PAS domain and a histidine kinase domain that couples sensing to signalling [6].

PASKIN - a potential oxygen sensing protein?

PAS domain proteins

Most of the PAS proteins are located in the cytoplasm and act as sensor modules for sensing changes in light (phytochromes), oxygen (FixL), redox potentials (Aer, NifL, ArcB), voltage-activated ion channels (HERG) and binding of certain ligands (AhR) for the detection of sensory input signals [7-9]. Unlike the PAS proteins of these two-component regulatory systems, PAS proteins also serve as heterodimerization interfaces of transcription factors involved in xenobiotic response (AhR/ARNT) [10], circadian rhythm (CLOCK/BMAL and PER) [11], carbon monoxide signalling (NPAS2/BMAL1) [12], as well as in the regulation of response to hypoxia (HIF1,2,3α/ARNT) [13]. PAS domains were identified in over 1100 proteins in archaea, bacteria and lower eukarya, but only relatively few sensor PAS proteins have been reported in mammalian species [4,14,15]. The term PAS is derived from the three founding members of the family - PER, ARNT and SIM. The PER protein of *Drosophila melanogaster* represents a central component in the periods of circadian clocks, ARNT is as an aryl hydrocarbon receptor nuclear translocator and SIM, a product of Drosophila single-minded gene, controls the CNS midline cell development (Fig. 3). The PAS domain of these three proteins shows a homologous region containing a 270 amino acid stretch of which two conserved 50 amino acid domains divided the sequence into PAS A and PAS B [7,8].
The first 3-dimensional structure of this widespread PAS protein fold was solved for the blue-light photoreceptor PYP (photoactive yellow protein) of the *Ectothiorhodospira halophila* that represents a structural prototype for the superfamily of all PAS proteins (Fig. 2). The typical structure is formed by five-stranded antiparallel β-sheets and several α-helices on one side. In Figure 2 three independent PAS-proteins HERG (human *eag*-related gene) a member of the ether a go-go potassium channel family, PYP and the oxygen-sensing protein FixL are shown. The three PAS domains are involved in signal transduction and reflect a high degree of structural similarity. The three PAS domain proteins are activated by different mechanisms. HERG is an ion channel and belongs to the voltage-gated channels controlled by membrane potential [16]. In that case no ligand binding is involved in the activation of HERG.

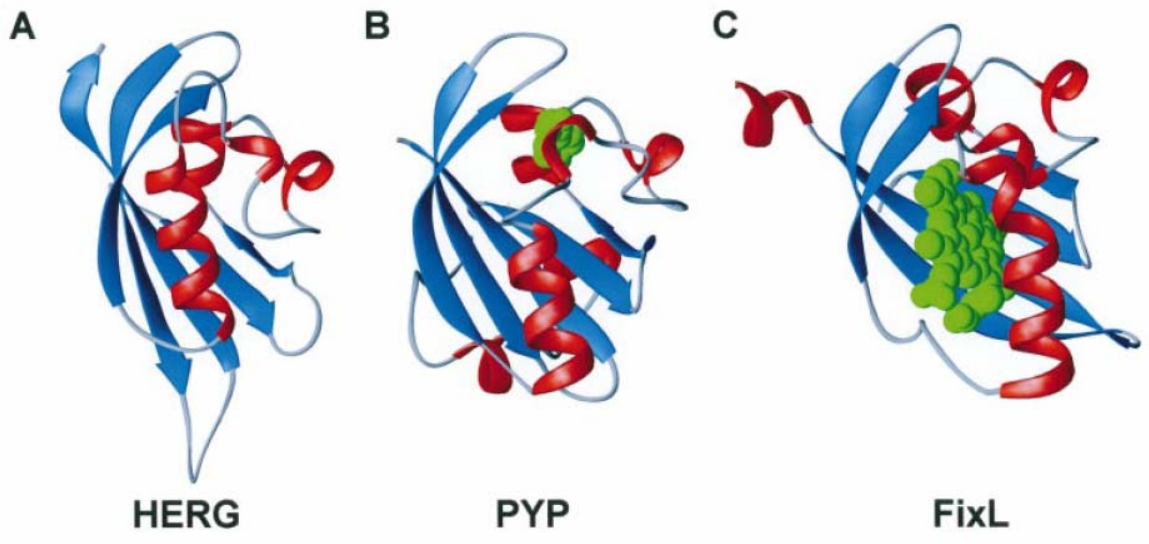

Figure 2: Schematic representation of HERG (A), PYP (B) and FixL (C) shows a high degree of structural similarity. β-strands are colored in blue, α-helices in red and cofactor ligands in green [17].

Unlike the HERG PAS domain, PYP as well as FixL are activated by certain ligands. The photoreceptor PYP contains a chromophore (4-hydroxycinnamic acid) that is covalently bound to a cysteine in the core of this protein. By photon absorption, an isomerisation takes place and results in conformational change of the protein surface [17,18]. The bound chromophore is not an activating ligand, but an essential co-factor for light perception.
In case of FixL the heme-cofactor binds to the PAS domain of this protein. The activity of the histidine kinase of FixL is controlled by binding of oxygen to the heme-group within the PAS domain.

PASKIN is related to the bacterial oxygen sensor FixL

The soil-nitrogen fixing bacteria, *Rhizobium* and *Bradyrhizobium* spezies, live in root nodules of certain plants, such as soybean, and exhibit a symbiotic association with its host. The nitrogen fixation of this bacterium is controlled by two kinases FixL and FixJ; acting as a sensor/regulator pair in a two-component system. At low oxygen concentrations, O_2 dissociates from the ferrous iron of the heme-bearing domain, resulting in activation of its kinase domain of FixL by autophosphorylation.
In the presence of ATP the autophosphorylated form of FixL phosphorylates the regulator protein FixJ that promotes the expression of nitrogen fixing genes *nif* and *fix* as transcriptional activator [6,19]. A spin-state mechanism is proposed for FixL. The kinase is activated in an unligand ferrous state (Fe^{2+}) of the bound heme; binding of ligands like oxygen, cyanide and

carbon monoxide inhibit the kinase activity [20]. Upon ligand binding, a conformational change takes place in the structure of the heme active site in a region between the F_{α}-helix and the G_{β}-strand, termed the FG loop.

PASKIN contains two PAS domains with higher sequence similarity to FixL than to any other known mammalian PAS protein. PASKIN is the first mammalian PAS protein that contains a kinase domain and hence could act as an oxygen sensor and signalling protein. However, the PAS domain of bacterially expressed PASKIN fragments did not contain a chromophore like is observed for purified FixL protein from bacteria [4]. Thus, PASKIN is most likely not an oxygen sensor.

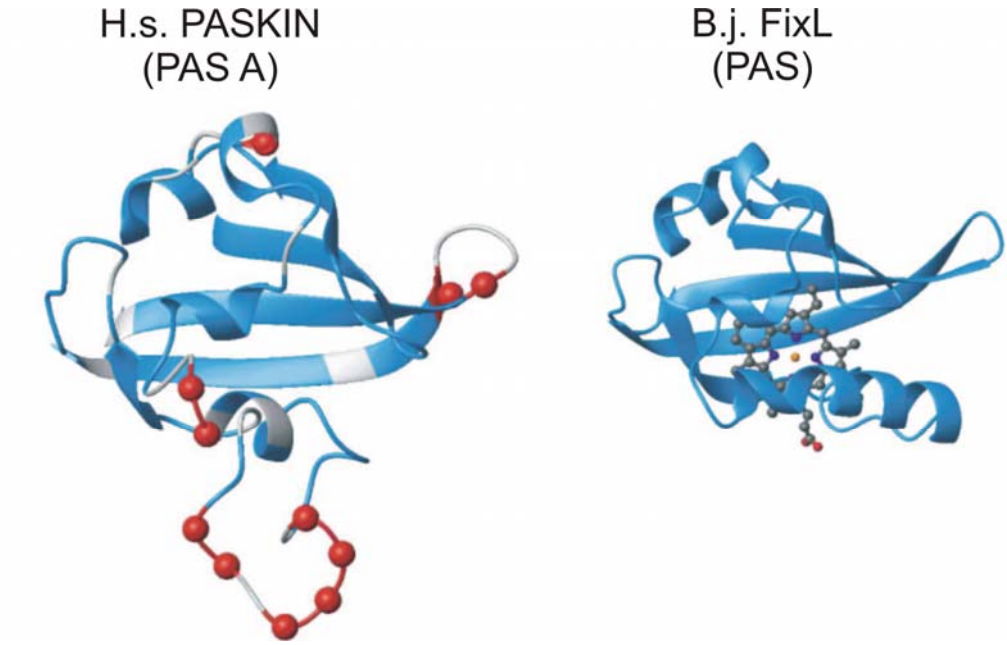

Figure 3: Three-dimensional structures of the PAS A domain of *Homo sapiens* (H.s.) PASKIN and the PAS domain of *Bradyrhizobium japonicum* (B.j.) FixL [15]. Amino acids indicated by red balls in the PAS A domain of PASKIN denote residues that undergo structural changes upon ligand binding. The iron-containing heme group of the oxygen-sensing FixL PAS domain is shown in the pocket of the PAS domain.

The 3-dimensional structure of the PASKIN PAS A domain has recently been resolved (Fig. 3) and synthetic ligands binding to this domain were identified by a nuclear magnetic resonance (NMR)-based screen of a library of over 750 organic compounds [15]. Binding compounds to PASKIN possess a hydrophobic character, containing one or two aromatic rings with polar groups and a linker. Both ligand binding as well as mutation of the PAS A domain result in the activation of the kinase domain. The synthetic ligands identified are structurally related to dioxin, known to bind to the PAS domain of aryl hydrocarbon receptor (AhR). However, an endogenous ligand of PASKIN has not been identified so far.

Structure and activation of PASKIN

PASKIN is encoded by a conserved single copy gene that is ubiquitously expressed. The gene structure of PASKIN consists of 18 exons (Fig.4). Interestingly, the analysis of the genomic structure of human *PASKIN* and mouse *Paskin* genes shows the first exon of the *PPP1R7* gene 1.0 kb upstream in the promoter region of PASKIN. *PPP1R7* encodes Sds22, a regulator of serine/threonine protein phosphatase-1 [21-23]. The open reading frame of human PASKIN cDNA encodes a protein with a predicted molecular weight of 144 kDa. PASKIN possesses two N-terminal consensus PAS repeats, PAS A and PAS B and a serine/threonine kinase in the C-terminal part of that protein. Unlike the high structurally conservation to the PAS domain of FixL, the histidine kinase domain weakly matched to exon 10 of PASKIN [4]. The amino acid sequence of the catalytic domain of PASKIN is highly related to Snf1 and AMPK protein kinases [5,24]. It has been shown that active PASKIN phosphorylates the synthetic AMARA peptide in the presence of ATP. This peptide is known as kinase substrate of Snf-1 and AMPK [25]

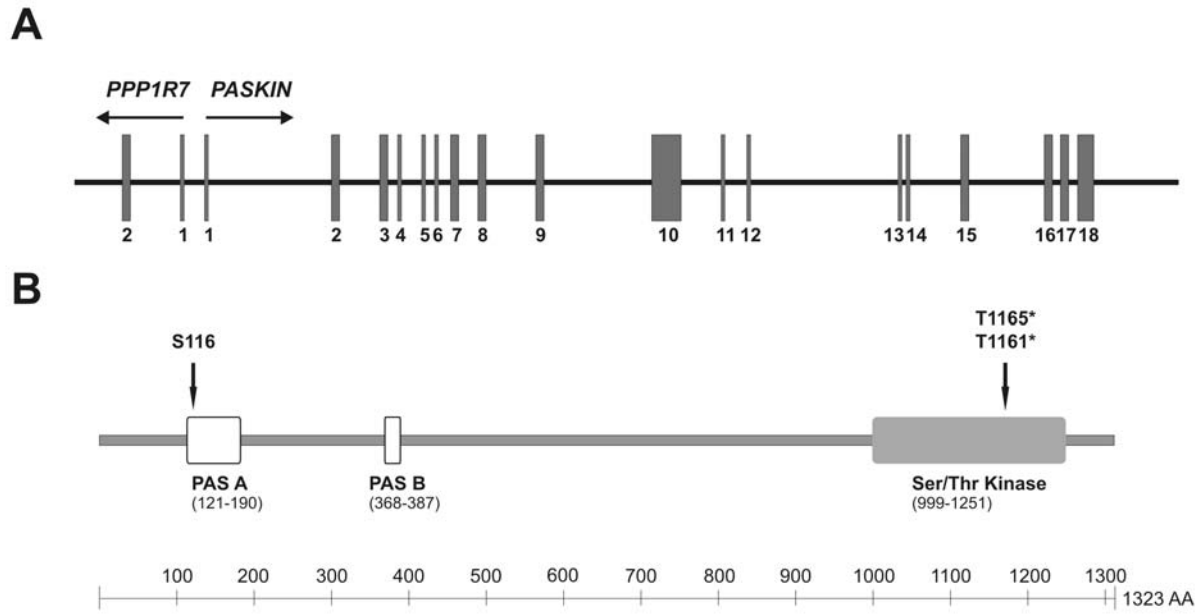

Figure 4: Primary structure of human PASKIN. (A) Gene structure of human *PASKIN*. Exons are indicated by grey boxes numbered according to the human sequence [4]. (B) Protein domain structure of PASKIN with known phosphorylation sites.

Two major autophosphorylation sites are located in the catalytic domain of PASKIN at Thr1161 and Thr1165. The Thr1161A mutant as well as the Thr1165 mutant of PASKIN had no detectable kinase activity, suggesting that both phosphorylation sites are necessary for efficient kinase activity of PASKIN [5]. Analogous phosphorylation patterns have been shown for the related AMP-kinase and its yeast homolog Snf1 [26,27]. A mapping of further phosphorylation sites on PASKIN resulted in two putative regions in the C-terminal part of PASKIN, comprising residues 1270-1286 and 1287-1318. Five serine residues (S1273,-77,-80 and S1287,-89) have been analysed by changing serine residues to alanine, but different variants of mutation show kinetic parameters indistinguishable from the native human PASKIN enzyme [5].

Another phosphorylation site (S116) was identified in the N-terminal region of PASKIN by a large scale characterization of HeLa cell nuclear phosphoproteins [28]. The function of PASKIN in the nucleus is unknown so far. Recently, it has been shown that PASKIN phosphorylates the pancreatic duodenal homeobox protein PDX-1 *in vitro* [29] and apparently inhibits its translocation to the nucleus, suggesting a functional role of PASKIN in the nucleus.

As known from FixL, the PAS A domain of PASKIN represses the kinase activity in *cis*. [5] The structural features of a PAS domain is a typical α/β PAS domain fold, characterized by several α-helices and five-stranded antiparallel β-sheets (Fig.3) [15]. NMR analysis provides portions of the flexible Fα helices and the FG loop region for a putative interaction site for binding and inhibition of the kinase domain [15].

An existing model of PASKIN activation proposes that in an unbound ligand state, an inhibition of the own kinase domain by binding to the FG loop takes place (Fig.5). Rutter and colleagues could show that the catalytic activity of the N-terminal mutant of human PASKIN was 5 fold higher than the full-length human PASKIN [5]. Additionally, by adding increasing amounts of purified PAS A domain, a decrease in the kinase activity takes place. The role of the PAS B domain in the regulation of kinase activity is unknown so far. De-repression, presumably by binding of small ligands in the hydrophobic core of the PAS A domain, leads to conformational changes in the FG loop and results in an autophosphorylation in *trans* [5]. As a consequence, the activated kinase is able to phosphorylate downstream targets of PASKIN in the cell. An endogenous ligand for PASKIN activation has not been identified so far.

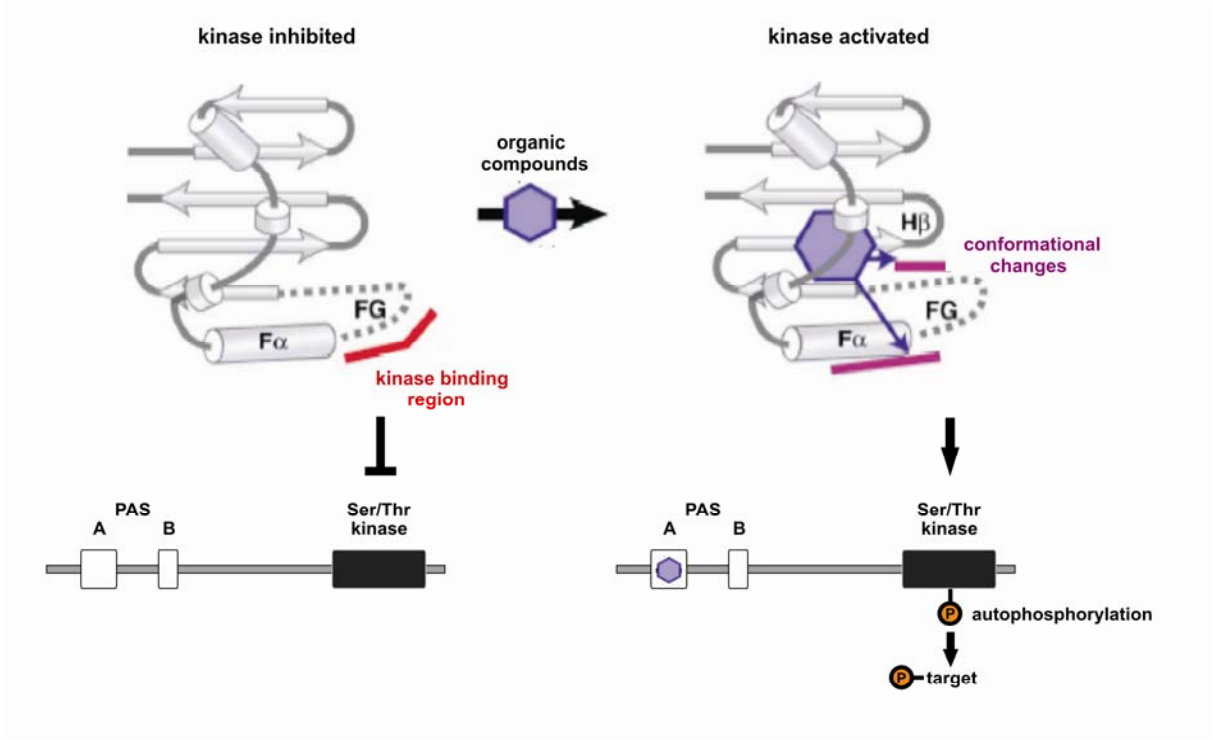

Figure 5: Hypothetic model for PASKIN activation by small ligands. No endogenous ligands are known so far. [15].

Kinase activation by phospholipids

A classical way to stimulate kinase activity has been shown for the protein kinase PKC which is activated by endogenous ligands such as phospholipids. PKC is a calcium- and phospholipid-dependent serine/threonine kinase that acts as a central mediator in the nervous system and other cell types [30]. PKC is involved in intracellular signals that regulate growth, differentiation and apoptosis in the cell. PKC isoenzymes are divided in three subgroups according to their structural homology and regulatory properties. The classical PKCs (α, βI, βII, γ) can be activated by calcium, diacylglycerol (DAG) and phosphatidylserine (PS). The novel PKCs (δ, ε, η, μ and θ) are calcium independent and can be also stimulated by DAG, PS and unsaturated fatty acids. Finally, enzyme activity of the atypical PKCs (ζ, λ and ι) is enhanced by PS, phosphatidylinositides and unsaturated fatty acids, but is unresponsive to calcium and DAG [31]. Two cysteine-rich domains in the N-terminal part of classical and novel PKCs were identified as binding site for DAG and phorbolesters, while phorbolesters mimic DAG action [32,33]. In the classical model for PKC activation the phospholipase C (PLC) is stimulated by G proteins or receptor mediated tyrosine kinases, cleaving PIP_2 at the inner plasma membrane to generate membrane-bound DAG and IP_3 that increase intracellular

calcium level. DAG and calcium cause the translocation of PKC from the cytosol to the plasma membrane (Fig. 6) [34].

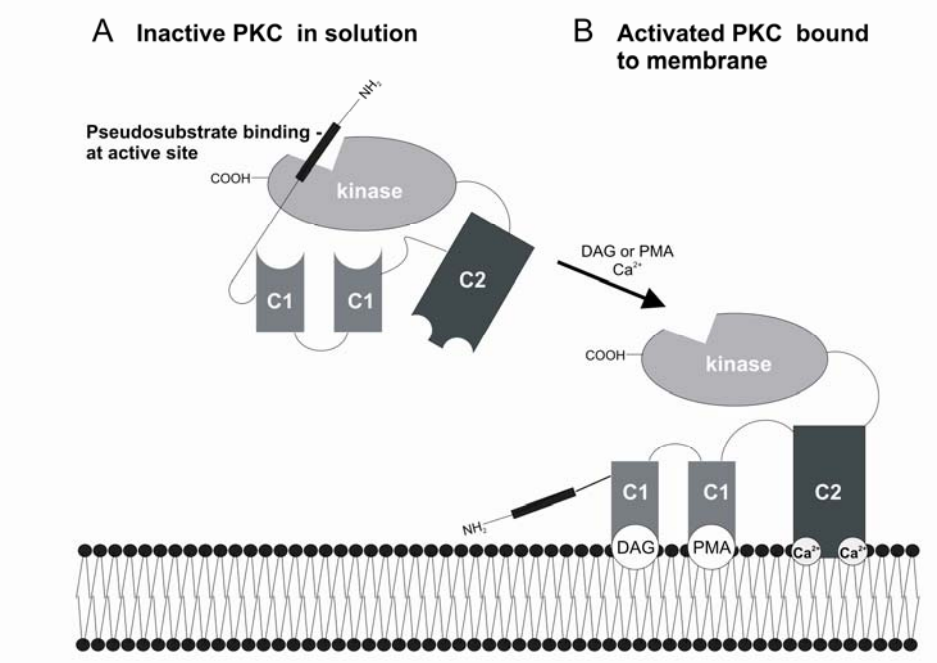

Figure 6: (A) Pseudosubstrate inhibits the catalytic domain of PKC (B) When the cysteine-rich C1 domains bind to diacylglycerol in the membrane, the pseudosubstrate is pulled from the active site, permitting catalysis. Calcium-binding C2 domains help to localize PKC to the membrane (modified from [35])

Hannun and Bell show that four or more molecules of PS bind cooperatively to PKC in combination with only one molecule DAG to maximally activate the serine/threonine kinase [36,37]. The unsaturated fatty acid ester groups of PS seem to be partially responsible for the activation of PKC. Neither phosphatidylethanolamine nor phosphatidylcholine alone shows a stimulation of PKC, but in a mixture with PS an enhanced enzyme activity is observed for PKC [38]. Phosphatidylinositol, phosphatidic acid and sphingomyelin are not able to stimulate PKC activity [39].

The protooncogene product Raf-1 is another lipid-dependent kinase, containing a cysteine-rich domain in the amino terminus of Raf-1 that is able to bind PS *in vitro* [40]. An interaction between Raf-1 and PS leads to a translocation of the protein to the membrane [41,42] and the binding of PS is not dependent on the classical PKC coactivators DAG or phorbolesters. In interaction studies from Gosh and colleagues showed that of all tested lipids phosphatidic acid and cardiolipin strongly interact with Raf-1. In vivo, the formation of PA by the phospholipase D (PLD) is inhibited by 1% ethanol that blocks the translocation of the Raf-1 kinase from the cytosol to the membrane [41].

Further proteins interacting with PS are involved in the retrieval of synaptic vesicle in nerve terminals (dynamin I) [43], necrosis (Hsp70 family) [44], specific hydrolysis of phosphatidylserine (phospholipase A1) [42] and stress–induced ceramide generation in the brain (neutral sphingomyelinase) [45,46].

Phospholipids

Phospholipids consist of four components: fatty acids, a negatively-charged phosphate group, alcohol and a backbone. The phospholipids are divided in two classes by the structure of their backbones: phosphoglycerides and sphingomyelin.

A phosphoglyceride is composed of fatty acyl side chains that are esterified to two hydroxyl groups in glycerol and the third hydroxyl group is esterified to phosphate. The phosphate group forms a connection by esterification to a hydroxyl group of another hydrophilic compound such as choline in phosphatidylcholine. Instead of choline, ethanolamine, serine, and the sugar derivative inositol are bound to phosphate in other phosphoglycerides.

Sphingomyelin is a phospholipid that contains instead of a glycerol backbone an amino alcohol with a long unsaturated hydrocarbon chain also termed sphingosine. The terminal hydroxyl group of sphingomyelin is esterified to a phosphocholine.

Phospholipids are characterized as amphipathic molecules containing a hydrophilic and a hydrophobic part. The negative charge on the phosphate as well as the charged group or hydroxyl group interacts strongly with water. In aqueous solution, phospholipids are able to form micelles, liposomes and lipid bilayers. The fatty acyl chains aggregate by hydrophobic interactions and exclude water molecules from the inner lumen of this aggregate [47].

Micelles form spontaneously with a hydrophobic interior composed entirely of fatty acyl chains. Unlike micelles, liposomes are arranged in a bilayer structure containing an aqueous center. A phospholipid bilayer, as is known from all biological membranes, has a hydrophobic core and separates two aqueous solutions [47]. The lipid composition of a typical nucleated mammalian cell is shown in table 1. The most abundant phospholipid is phosphatidylcholine with 40-50% of total phospholipids in the eukaryotic cell. Phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin are also present in significant amounts. The composition of phospholipids varies in different tissues and different cell types. Phosphatidylserine is enriched more than 36% in the brain compared to other tissues. [48] Lower levels of PS are present in mitochondrial inner membranes and lysosomes than in other membranes such as the endoplasmic reticulum, nucleus and plasma membrane.

Interestingly, phosphatidylserine is the most abundant protein in the cell membrane in yeast comprising 34% of all phospholipids. The biological significance of this enrichment in yeast is unknown so far. Unlike PS, phosphatidylethanolamine is more abundant in mitochondria than in other tissues [46].

Lipid	% Total lipids
Phosphatidylcholine	45–55
Phosphatidylethanolamine	15–25
Phosphatidylinositol	10–15
Phosphatidylserine	2–10
Phosphatidic acid	1–2
Sphingomyelin	5–10
Cardiolipin	2–5
Glycosphingolipids	2–5
Cholesterol	10–20

Table 1: Lipid composition of typical nucleated mammalian cell [46]

Phospholipases such as PLC and PLD are enzymes that play an important role in the formation of phospholipids hydrolyzing various bonds in the head group of phospholipids [47]. Extracellular signals such as PMA (phorbol 12-myristate 13- acetate) often activate PLD [49,50]. The mechanism how PMA activates PLD *in vivo* is so far unknown [51]. It has been shown *in vitro* that PKC is able to interact with PLD and activates it in an ATP-independent manner [52]. PLD generates phosphatidic acid by hydrolysis of phosphatidylcholine or other phospholipids [53-55]. Phospholipids are able to interact with enzymes and can stimulate kinase activity resulting in downstream-target activation such as it is known for Raf kinases.

The functional role of PASKIN

Targets of PASKIN in yeast

The first insights into target identification of PASKIN were obtained for the yeast PASKIN homologs PSK1 and PSK2. Under stress conditions, the psk1 psk2 double-mutant strain shows a decreased growth at elevated temperature (39°C) and galactose supply. Two enzymes have been identified which are involved in the regulation of glycogen and trehalose synthesis. The UDP-glucose pyrophosphorylase (Ugp1) catalyzes the conversion of glucose-1-P to UDP-glucose which is inhibited by Psk2 phosphorylation. The other enzyme the glycogen synthase 2 (Gsy2), adds glucose monomers to glycogen by release of UDP and is also inhibited by efficient phosphorylation by Psk2. The negative regulation of Ugp-1 and Gsy2

suggests that PASKIN plays a role in the switch of glycogen storage in favour of glycolysis [56]. Recently, it has been shown that the mammalian glycogen synthase interacts with the midregion of human PASKIN and is phosphorylated at Ser-640 [57].

A second biological function of PASKIN in yeast is based on the identification of three further phosphorylation targets in yeast: Caf20, Tif11 and Sro9. They promote several steps of protein synthesis. The Cap-associated factor 20 (Caf20) is a small protein termed p20 that blocks the association of the mRNA cap-binding protein, eukaryotic translation initiation factor eIF4E and eIF4G. p20 and eIF4G bind competitively to eIF4E [58]. Rutter et al. show that Caf20 in complex with eIF4E was efficiently phosphorylated by Psk2, but interestingly neither of them was phosphorylated alone by Psk2 [5].

The second phosphorylation target Tif11 is the yeast ortholog of eukaryotic translation initiation factor 1A (eIF1A) that mediates the transfer of aminoacyl-tRNA to the 40S ribosomal subunit [59].

The third Psk2 substrate, the RNA binding protein Sro9, is associated with translation-active ribosomes [60] and is also involved in the organization of actin filaments [61]. In summary, the yeast PASKIN homologs phosphorylate three translation factors and two enzymes involved in the regulation of glycogen and trehalose synthesis, thereby coordinately controlling translation and sugar flux [56].

High expression of PASKIN during spermatogenesis

To elucidate the function of mammalian PASKIN, our lab previously inactivated the mouse *Paskin* gene by homologous recombination in embryonic stem cells [14]. The replacement of exon 10 to 14 by a *lacZ* reporter gene was used for the identification of cell types expressing mouse PASKIN by X-Gal staining. *Paskin*$^{-/-}$ mice showed normal development, growth and reproduction. By Northern blot analysis of mouse organs PASKIN mRNA expression was 90-fold higher in testis than in the thymus, the organ with the second highest PASKIN mRNA expression. Very low levels of PASKIN mRNA expression were observed for the other tissues such as the brain, lung and liver [14].

Da Silva Xavier et al. suggested that PASKIN kinase activity, mRNA and protein expression is upregulated in MIN6 cells and in isolated pancreatic β-cells after treatment with high glucose concentrations [62]. In a recent report, our lab could show that PASKIN mRNA induction is not induced in MIN6 or in pancreatic β-cells. Increasing glucose concentrations lead to induction of insulin mRNA levels and insulin release, but independent of PASKIN

mRNA expression or protein levels. Analysis of blood glucose levels and glucose tolerance test show no differences between *Paskin* wildtype and knock out mice [63]. X-Gal stainings for β-galctosidase reporter gene activity showed a high PASKIN level in the seminiferous tubuli of mouse testis [14,63]. No other tissue, including ovary and uterus, revealed any blue color staining, suggesting that the testis is the main organ for PASKIN expression. X-Gal stainings and in situ hybridization by silver grain visualization of mouse testis section revealed PASKIN expression in spermatocytes, spermatids and spermatozoa, but not in spermatogonia or Leydig cells. PASKIN expression is strongly upregulated in postmeiotic spermatids suggesting an involvement of PASKIN in sperm cells after leaving the testis. However, the absence of PASKIN affects neither male fertility nor sperm production or motility [14].

Downstream targets of PASKIN in mammals

The eukaryotic translation elongation factor eEF1A1

To get more insight into the function of PASKIN, we identified eEF1A1 as protein-protein interaction partner of PASKIN in a yeast two-hybrid screening with the PAS sequence of PASKIN as bait and a HeLa cDNA library elongation factor. eEF1A1 is an ubiquitously expressed GTP-binding protein. Another isoform, eEF1A2, is highly expressed in the heart, brain and skeletal muscle, but was not detectable in other tissues like liver, lung, kidney, placenta and pancreas [64]. Interestingly, eEF1A1 is barely expressed in heart and skeletal muscle, suggesting a tissue-dependent variation in the expression of both isoforms [65]. eEF1A is one of many different factors which are involved in the elongation step of protein biosynthesis. The elongation cycle of the protein biosynthesis is shown in figure 7. There, eEF1A in complex with GTP and aminoacyl-tRNA mediates the binding of the tRNA to the A-site of the ribosome. Upon placement of the aminoacyl-tRNA, GTP hydrolysis takes place and results in the dissociation of eEF1A and GDP from the ribosome. The eEF1B complex (α, β, γ) stimulates the conversion of the inactive form of eEF1A by a GDP/GTP exchange, starting another round of tRNA binding and recruitment. In the translocation phase of protein synthesis, first a peptide bound is formed between the amino acids at the P and A site of the ribosome. In a second step, eEF2 mediates the translocation of the peptidyl-tRNA from the A site to P sites by hydrolysing GTP. The previous peptidyl-tRNA changes from the P to the E site and is finally released. The ribosome moves the distance of three nucleotides of

the mRNA to reach the next codon. Upon release of eEF2-GDP the translocation process is finished and the A site is free for a new aminoacyl-tRNA transferred by eEF1A.

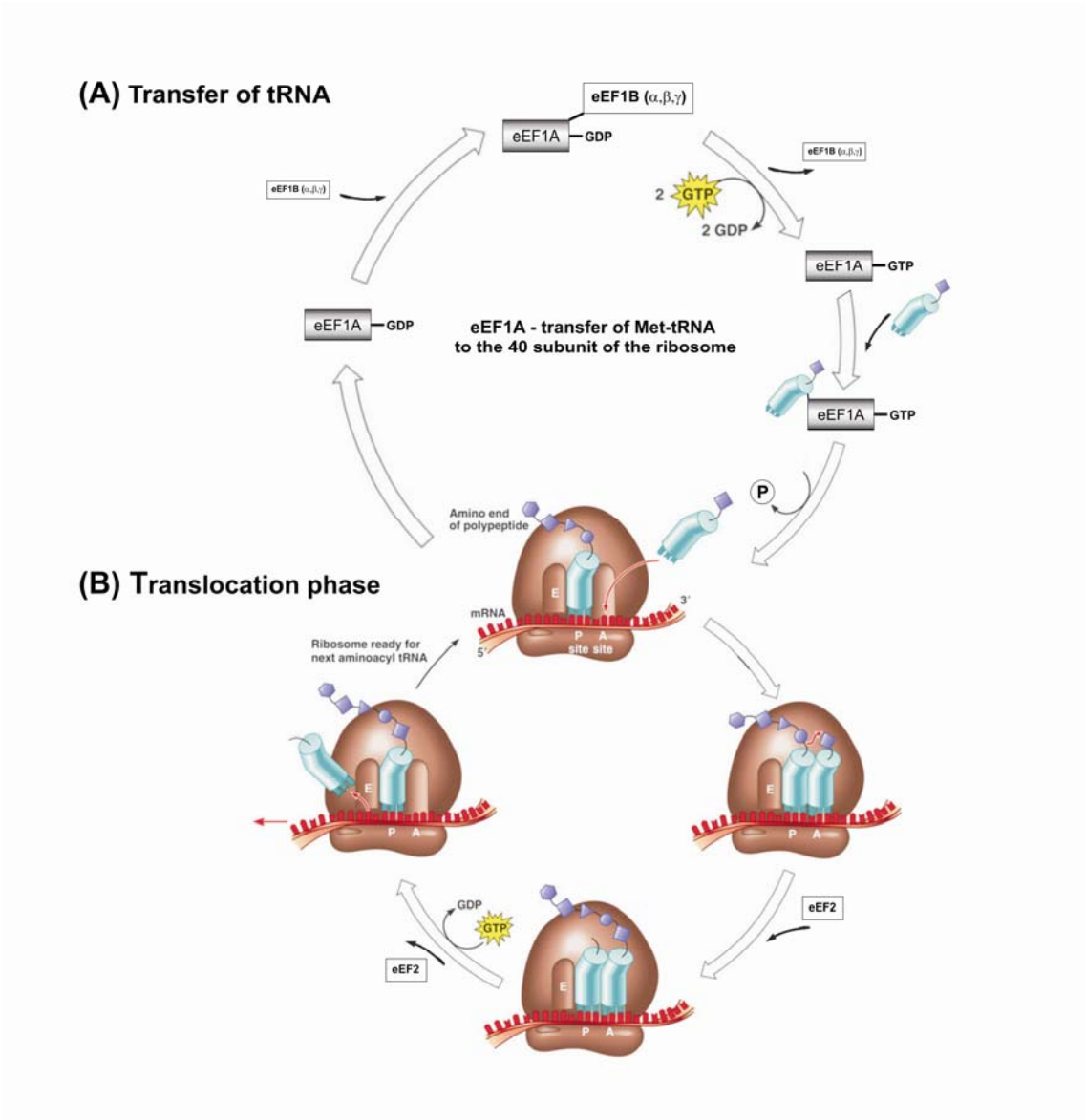

Figure 7: Scheme of the elongation cycle of eukaryotic protein synthesis. (A) eEF1A transfers aminoacyl-tRNA to the A-site of the ribosome. GTP hydrolysis allows eEF1A to dissociate from the ribosome and bind to the eEF1B complex. GTP/GDP exchange is mediated by this complex starting another round of recruitment of tRNA. (B) A peptide bound is formed between the amino acids from the P and A sites. eEF2 binds at the A site of the ribosome and induces the translocation of three nucleotides towards 3'-end of the mRNA by hydrolysis of GTP. After dissociation of eEF2 from the ribosome, the A site is left free for a new transferred tRNA (modified from [66]).

Insulin and phorbol esters are able to stimulate the phosphorylation of eEF1A1 *in vivo*. [67,68]. Chang and colleagues have shown that the multipotent S6 kinase is primarily responsible for the phosphorylation of eEF1A1 after insulin treatment [69]. In response to the tumor promoting phorbol ester PMA, eEF1A1 is phosphorylated by PKC *in vitro* and *in vivo* [67,68]. PKC isoforms such as PKCα, β and γ are also able to phosphorylate eEF1A1, but PKCδ was the most efficient isoform of all tested PKCs. Kielbassa et al. identified PKCδ phosphorylation of eEF1A1 at Thr431 [70].

In addition to protein synthesis, eukaryotic EF1A1 has been shown to be involved in other cellular processes such as cell growth, stress response or motility [71]. An involvement of eEF1A in phosphoinositide pathway is confirmed by an interaction with PLCγ–1 *in vitro* and *in vivo*. The interaction partner was found by yeast two-hybrid screening of a human B-lymphocyte library. After EGF treatment, the interaction between eEF1A and PLC-γ1 was increased, suggesting an involvement of eEF1A in PLCγ-mediated signal transduction. [72]

Furthermore, eEF1A was identified as a phosphorylation target of the plant chimeric calcium / calmodulin-dependent protein kinase (CCaMK) that phosphorylates a threonine residue within a region of the putative aminoacyl-tRNA binding site in a calcium- and calmodulin-dependent manner [73]. The phosphorylation of eEF1A by CCaMK has been shown to reduce actin-bundling activity [74]. Interactions of eEF1A with the actin cytoskeleton were first observed in *Dictyostelium amoebae* and this function could be confirmed across species from yeast to mammals. In yeast, the actin bundling by eEF1A is not necessary for translation, unlike the mammalian system where a disruption of actin cytoskeleton leads to a reduced translation [75,76]. These observations suggest a different function of actin in different eukaryotic species [77]. A link between eEF1A and actin cytoskeleton has also been shown in cancer, supporting the requirement of the actin cytoskeleton for metastasis [78]. Eukaryotic EF1A mRNA expression is highly upregulated in human pancreas, colon, breast, lung and gastric tumors [79]. Increasing amounts of eEF1A protein expression were observed in metastatic tumor cells leading to a reduced binding to F-actin. After stimulation with epidermal growth factor (EGF) F-actin as well as eEF1A protein expression was remarkably enhanced, indicating a redistribution of EF1A into the actin cytoskeleton in tumor cells [78].

The eukaryotic EF1A seems to be also involved in apoptosis. Studies with a temperature-sensitive mutant of p53 have shown an upregulation of eEF1A [80]. After treatment with hydrogen peroxide, increased levels of endogenous eEF1A were observed in cardiomyocytes, suggesting an involvement of eEF1A in apoptosis in response to oxidative stress [81,82].

The DNA-repair protein Ku70 as a putative interaction partner of PASKIN

To verify the functional role of PASKIN in the testis, a second yeast two-hybrid screening with a human testis cDNA library and the kinase domain of PASKIN as bait revealed the DNA repair protein Ku70 as an interaction partner of PASKIN.

Ku70 is a multifunctional protein mainly involved in DNA-repair. Numerous reports implicate Ku70 in other cellular processes, including V(D)J recombination, antigen-receptor-gene rearrangements, mobile-genetic element biology, telomere maintenance, apoptosis and transcription [83]. It has been reported that the Ku complex is localised in not only nuclei but also cytoplasm [84-86]. In eukaryotes, Ku is a stable heterodimer consisting of two subunits with 70 and 80 kDa, named Ku70 and Ku80. Each Ku subunit possesses a von Willebrand A domain (vWA) in the N-terminus and a central core domain (Fig. 8).

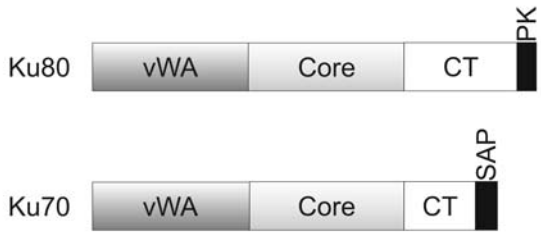

Figure 8: Domain organization of Ku80 and Ku70 protein. Ku proteins consist of a von Willebrand A domain (vWA), a central core domain. In the C-terminus, Ku80 contains a binding site for DNA-PKcs and Ku70 possesses a SAP motif with an unknown function [83].

The von Willebrand A domain acts as protein-protein interaction site. Interestingly, Ku70 has a SAP domain in the carboxy-terminal region which is named after three proteins containing a typical SAF-A/B, Acinus and PIAS motif. This motif is derived from a helix-extension-helix fold [87]. The function of this domain is unknown so far. In Ku80 the carboxy-terminal region is longer and contains a binding site for DNA-PK_{CS} [83]. A model for the formation of the Ku-DNA-DNA-PK complex suggests that after heterodimerization of Ku70 and Ku80 the DNA is bound in an asymmetric ring of the Ku-complex [88] and creates a binding substrate for DNA-PK_{CS}. In this Ku-DNA complex the kinase activity of DNA-PK_{CS} is stimulated and it phosphorylates downstream targets that are involved in DNA-repair such as tumor suppressor protein p53, Fos, Sp1, H2AX and RNA-polymerase II [89,90].

The autophosphorylation of DNA-PK_{CS} leads to a dissociation of DNA-PK_{CS} from Ku-DNA complex [83].

In conclusion, in mammals the PDX-1, the glycogen synthase, eEF1A1 as well as the putative interacting protein Ku70 were identified as downstream targets of PAKSIN. The above discussed targets of PASKIN in yeast and mammals suggest a possible role of PASKIN as multifunctional protein that is involved in many cellular processes such as protein translation, sugar flux, apoptosis and actin cytoskeleton bundling.

Goal of this thesis

The goal of this thesis was to identify novel interacting proteins derived from the testis which interact with PASKIN. An initial yeast two-hybrid screening with a HeLa cDNA library revealed the eukaryotic translation elongation factor EF1A1 as a novel interaction partner of PASKIN. To obtain more insights into the functional role of PASKIN during the spermatogenesis, we performed a second yeast two-hybrid screening with a human testis cDNA library. The discovered protein-protein interactions of PASKIN with emphasis on the eukaryotic translation elongation factor eEF1A1 have to be confirmed *in vitro* and functional assays are required. It is known that synthetic ligands bind to PASKIN, but an endogenous ligand for PASKIN activation is unknown so far. Therefore, another goal of the thesis was to identify endogenous ligands that trigger PASKIN stimulation, leading to the activation of target phosphorylation. The identification of such an activator would be of high importance in the studies of PASKIN characterization.

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5. MALE GERM CELL EXPRESSION OF PASKIN AND ITS NOVEL TARGET eEF1A1

Male germ cell expression of the PAS domain kinase PASKIN and its novel target eukaryotic translation elongation factor eEF1A1

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Key words: Energy homeostasis, Glycogen synthesis, Nitrogen fixation, Protein phosphorylation, Protein translation, Testis

ABSTRACT

PASKIN links energy flux and protein synthesis in yeast, regulates glycogen synthesis in mammals, and has been implicated in glucose-stimulated insulin production in pancreatic β-cells. Using newly generated monoclonal antibodies, PASKIN was localized in the nuclei of human testis germ cells and in the midpiece of human sperm tails. A speckle-like nuclear pattern was observed for endogenous PASKIN in HeLa cells in addition to its cytoplasmic localization. By yeast two-hybrid screening, we identified the multifunctional eukaryotic translation elongation factor eEF1A1 as a novel interaction partner of PASKIN. This interaction was mapped to the PAS A and kinase domains of PASKIN and to the C-terminus of eEF1A1 using mammalian two-hybrid and GST pull-down assays. Kinase assays, mass spectrometry and site-directed mutagenesis revealed PASKIN auto-phosphorylation as well as eEF1A1 target phosphorylation mainly but not exclusively at Thr432. Wild-type but not kinase-inactive PASKIN increased the *in vitro* translation of a reporter cRNA. Whereas eEF1A1 did not localize to the nucleus, it co-localizes with PASKIN to the cytoplasm of HeLa cells. The two proteins also showed a remarkably similar localization in the midpiece of the sperm tail. These data suggest regulation of eEF1A1 by PASKIN-dependent phosphorylation in somatic as well as in sperm cells.

INTRODUCTION

Physiological adaptations of an organism to changing environmental conditions require molecular sensors capable of sensing and signalling specific physico-chemical parameters. The PAS (Per-Arnt-Sim) domain is a widespread protein fold of environmental protein sensors involved in the perception of light intensity, oxygen partial pressure, redox potentials, voltage and certain ligands [1]. In mammals, the PAS domain is mainly found as a heterodimerization interface of transcription factors involved in the molecular circadian clock, dioxin toxicity and oxygen sensing [2-4].

We and others previously identified a novel mammalian PAS protein, termed PASKIN [5] or PAS kinase [6]. The domain architecture of PASKIN resembles that of the oxygen sensor protein FixL from nitrogen-fixing *Rhizobium* species. PASKIN contains two PAS domains (PAS A and PAS B) and a serine/threonine kinase domain related to AMP kinases which might be regulated in *cis* by binding of so far unknown ligands to the PAS domain [7].

Following de-repression, autophosphorylation in *trans* results in the "switch-on" of the kinase domain of PASKIN [6]. The budding yeast PASKIN homologs PSK1 and PSK2 phosphorylate three translation factors and two enzymes involved in the regulation of glycogen and trehalose synthesis, thereby coordinately controlling translation and sugar flux [8]. Under stress conditions (nutrient restriction combined with high temperature), PASKIN kinase activity results in increased protein synthesis and decreased carbohydrate storage in yeast. PASKIN-dependent phosphorylation also inhibits the activity of the mammalian glycogen synthase [9]. In addition, a recent report suggested that PASKIN expression as well as kinase activity is increased in isolated pancreatic β-cells following stimulation with high glucose levels [10]. Increased PASKIN activity appeared to be required for glucose-dependent transcriptional induction of preproinsulin gene expression, which might be related to PASKIN-dependent regulation of the nuclear import of pancreatic duodenal homeobox-1 transcription factor [11].

We recently generated PASKIN null mice by targeted replacement of the kinase domain of the mouse *Paskin* gene by a *lacZ-neo* fusion construct in embryonic stem cells [12,13]. Surprisingly, PASKIN expression is strongly upregulated in post-meiotic germ cells during spermatogenesis as revealed by *in situ* hybridization, β-galactosidase staining and mRNA blotting. In fact, PASKIN mRNA levels in testis are several magnitudes higher than in all other organs tested. However, at least under laboratory conditions, fertility as well as sperm production and sperm motility were not affected in PASKIN knock-out mice. No other organs, including pancreas, stained positive for β-galactosidase, and we could not detect any PASKIN-dependent insulin regulation [14].

To obtain more insights into PASKIN function, we generated specific monoclonal antibodies derived against PASKIN and screened a HeLa cDNA expression library in a yeast two-hybrid system to identify novel PASKIN interaction partners in mammals. Here we show that the eukaryotic translational elongation factor eEF1A1 interacts with PASKIN. eEF1A1 is a GTP-binding protein catalyzing the binding of charged aminoacyl-tRNA to the A-site of the ribosome [15-17]. eEF1A1 is of particular interest because it is related to the yeast translation initiation factor eIF1A that mediates the transfer of Met-tRNA to the 40S subunit of the ribosome, and which was shown to be regulated by the yeast PASKIN homologs PSK1 and PSK2 [8].

MATERIALS AND METHODS

Plasmids

If not indicated otherwise, cloning work was carried out using Gateway technology (Invitrogen, Basel, Switzerland). pcDNA3hPASK [6], containing wild-type or mutant human PASKIN cDNA (kindly provided by J. Rutter, Salt Lake City, UT, USA), was digested with *Nco*I (all restriction enzymes were purchased from MBI Fermentas, Labforce, Nunningen, Switzerland), blunted with Klenow polymerase and subcloned into *Sal*I-*Eco*RV-blunted pENTR4 (Invitrogen) to obtain pENTR4hPASK. The PAS domain plasmid pENTR4PAS was obtained by *Bam*HI digestion and re-ligation of pENTR4hPASK. The kinase (KIN) domain plasmid pENTR4KIN was obtained by subcloning the *Ecl*136II fragment of pcDNA3hPASK into the *Xmn*I-*Eco*RV sites of pENTR4. Other fragments of PASKIN were amplified by PCR using Pfu polymerase (MBI Fermentas), digested with *Nco*I and *Xho*I and subcloned into the same sites of pENTR4. The following primers (synthesized by Microsynth, Balgach, Switzerland) were used: PAS A (5'-catgccatggtaagtgtgtcctgctgctccct-3' and 5'-ctagctcgagttactgccgcatcctcttcatcc-3'); PAS B (5'-catgccatggcttgggtgttctgcaccatc-3' and 5'-ctagctcgagttaggccaggtctgggagctgta-3'); N-terminal (5'-caggacgcccgccataaact-3' and 5'-ctagctcgagttatgaggaccaccctg-3'); centerpiece (5'-catgccatggagatccgaaagctgatggaa-3' and 5'-ctagctcgagttactcagcagcggtagagtgg-3'). pENTR4PASA1/2 was obtained by digesting pENTR4PASKIN with *Bam*HI and *Hin*dIII, Klenow fill-in and re-ligation. The plasmid pCMV6-XL5-eEF1A1, containing the full-length human eEF1A1 cDNA, was purchased from OriGene (Rockville, MD, USA). eEF1A1 was amplified by PCR (full-length: 5'-catgccatgggaaaggaaaagactcatatc-3' and 5'-ctagctcgagccgttcttccaccactgatt-3'; 1-241: 5'-aagcagaaggccatcctgac-3' and 5'-ctagctcgagttatggacgagttggtggtagga-3'; 247-462: 5'-catgccatggtgcgcctgcctctccaggat-3' and 5'-gatatctcgagccgttcttccac-3') and subcloned into pENTR4 as above. The T432A mutation was introduced by Pfu polymerase-based site-directed mutagenesis using the primer 5'-cgtgatatgagacaggctgttgcggtgggtg-3', followed by *Dpn*I digestion of the parental strain. The inserts of all ENTRy vectors were verified by DNA sequencing (Microsynth). To generate expression vectors for fusion proteins, ENTRy vectors were recombined *in vitro* with DESTination vectors using LR Clonase recombination enzyme mix (Invitrogen). pDEST15 and pDEST17 were used to generate bacterial or rabbit reticulocyte lysate expression vectors for GST- and His_6-fusion proteins, respectively. pDEST10 and pDEST20 were used to generate expression vectors for His_6- and GST-fusion proteins, respectively, in the baculovirus/Sf9 insect cell system (Invitrogen). pcDNA3.1/nV5-

DEST was used to express N-terminal V5-tagged proteins in mammalian cells or in rabbit reticulocyte lysates.

Generation of monoclonal antibodies

GST-PAS fusion protein expression was induced in *E. coli* BL21-AI by 0.2% arabinose for 4 hours and affinity purified with glutathione sepharose (Amersham Biosciences, Dübendorf, Switzerland). Two mice were immunized with this antigen using standard procedures. Hybridoma cell lines were established and culture supernatant tested against the antigen, or GST alone, by ELISA and immunoblotting. Antibodies from positive hybridoma supernatants were purified using protein A agarose (EconoPac protein A cartridge, BioRad, Reinach, Switzerland) liquid chromatography. Antibody isotyping was performed using ISOstrip mouse strips (Roche Diagnostics, Mannheim, Germany).

Immunoblotting

Combined cytoplasmic and nuclear extracts of cultured cells were prepared using 0.4 M NaCl and 0.1% NP-40 in extraction buffer as described previously [18]. Nuclear extracts were prepared from isolated nuclei using 0.4 M NaCl. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard. For immunoblot analysis, cellular protein was electrophoresed through SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by semi-dry blotting. Membranes were stained with Ponceau S (Sigma) to confirm equal protein loading and transfer. The following antibodies were used: mouse monoclonal antibody (mAb) anti-(α)Gal4 and mAb αVP16 (Santa Cruz Biotechnology, Heidelberg, Germany); mAb αeEF1A1 (Stressgen, Biomol, Hamburg, Germany); mAb αV5 tag (Invitrogen); mAb αHis_6 tag (Abcam, Cambridge, UK); rabbit polyclonal α β-actin (Sigma); and secondary polyclonal goat anti-mouse or anti-rabbit antibodies coupled to horseradish peroxidase (Pierce, Perbio, Lausanne, Switzerland). Chemiluminescence detection was performed by incubation of the membranes with 100 mM Tris-HCl pH 8.5, 2.65 mM H_2O_2, 0.45 mM luminol and 0.625 mM coumaric acid (all purchased from Sigma, Buchs, Switzerland) for 1 minute followed by exposure to X-ray films (Fuji, Düsseldorf, Germany). Alternatively, the Supersignal West

Dura kit (Pierce) was used and chemiluminescence was recorded with a CCD camera (FluorChem8900, AlphaInnotech, Witec, Littau, Switzerland).

Immunohistochemistry

Paraffin-embedded human testis samples (tumor orchidectomy) were sectioned (2 μm) and antigen retrieved by boiling the sections in 0.1 M citrate buffer for 90 minutes. Peroxidase activity was blocked by incubating with 3% H_2O_2 and unspecific binding sites were blocked with protein-block (Dako, Hamburg, Germany). The slides were incubated with αPASKIN mAb2 or mAb6, or αeEF1A1 mAb, diluted 1:50 and 1:100, respectively, in 50 mM Tris-HCl pH 7.6, 0.5 M NaCl, 0.1% Tween 20, 10% FCS for 15 minutes. Following washing, primary antibodies were detected with the CSA enhancing system (Dako) and DAB (for PASKIN) or FastRed (for eEF1A1) substrates. PASKIN production in SF9 was detected as described above except that the Envision system (Dako) with FastRed as chromogen was employed. All slides were counterstained with hemalaun.

Immunofluorescence

Collection of human ejaculated spermatozoa was approved by the ethics committee of the University of Leipzig (approval number 067-2005). Spermatozoa were washed in PBS, streaked onto microscope slides, incubated for 5 minutes in demembranization buffer (2% Triton-X100, 5 mM DTT, 50 mM Tris-HCl pH 9.0) and fixed with 3% paraformaldehyde. The non-specific binding sites were blocked with 3% BSA in PBS for 30 minutes. Spermatozoa were incubated for 1 hour with αPASKIN mAb2 or mAb6 diluted 1:10 in 3% BSA in PBS followed by a TexasRed-coupled secondary anti-mouse antibody (Dako) or an AlexaFluor 488-coupled secondary anti-mouse antibody (Invitrogen) diluted 1:100 with 3% BSA in PBS. HeLa cells were grown on cover slips and treated accordingly. Finally, nuclei were stained with Hoechst33258 dye for 5 minutes. After extensive washings with PBS, the slides were mounted and analyzed by fluorescence microscopy (Axioplan 2000, Carl Zeiss Vision, Mannheim, Germany) or confocal microscopy (LSM510, Carl Zeiss Vision).

Cell culture, transient transfection and co-immunoprecipitation

Human Hep3B hepatoma, HeLa cervical carcinoma and NCCIT male germ tumor (kind gift of S. Schweyer, Göttingen, Germany) cell lines were cultured in high glucose Dulbecco's modified Eagle's medium (Sigma) as described previously [19]. Transient transfection was performed with the polyethylenimine (PEI; Polysciences, Warrington, PA, USA) method. Therefore, 3 μg DNA in 100 μl 150 mM NaCl was mixed with 15 μl 1 mg/ml PEI stock solution in H_2O and 85 μl 150 mM NaCl. Following 30 minutes incubation at room temperature, the mixture was added to 50% confluent cells on a 10 cm dish and incubated for 24 hours. As determined by transient transfections with the green fluorescent protein expression vector pEGFP-C1 (Clontech, BD Biosciences, Heidelberg, Germany), transfection efficiency usually was 60-80% with this method (data not shown). For co-immunoprecipitation, pre-cleared cell extracts were incubated with αPASKIN mAb6 covalently coupled to sepharose A beads [20]. The beads were washed with 0.5% NP-40, 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM EDTA and bound proteins were analyzed by immunoblotting.

Yeast two-hybrid

A HeLa cell-derived cDNA library was screened using the PAS domain of human PASKIN as bait according to the instructions provided by the manufacturer (Clontech). Therefore, the full-length human PASKIN cDNA clone pDKFZp434O1522 [5] was digested with *Nco*I and *Sma*I and the cDNA fragment inserted into the *Nco*I-*Sma*I sites of the pAS2 vector (Clontech). Subsequently, the C-terminal part was deleted by *Bam*HI digestion and re-ligation to obtain pAS2PAS.

Mammalian two-hybrid

The mammalian Matchmaker vectors pM and pVP16 (Clontech) were converted to DESTination vectors by ligation of the Gateway vector conversion cassette reading frame B (Invitrogen) into the *Eco*RI sites (blunted with Klenow polymerase) of pM and pVP16 to obtain pMGAL4BDattb and pVP16ADattb, respectively. Mammalian expression vectors for Gal4 DNA-binding domain (BD) or VP16 activation domain (AD) fusion proteins were obtained after *in vitro* recombination with the corresponding ENTRy vectors. The pG5FL firefly luciferase reporter gene vector was obtained by replacing the *Sal*I-*Xba*I SEAP cDNA fragment from pG5SEAP (Clontech) with the *Sal*I-*Xba*I luciferase cDNA amplified by PCR (primers 5'-gatccgtcgactctagcatggaagacgccaaaaaca-3' and 5'-gctctagaattacacggcgatctttcc-3') from pGL3Basic (Promega, Madison, WI). Hep3B cells were transiently co-transfected with the BD and AD fusion protein vectors, the firefly luciferase reporter vector and the pRL-SV40 renilla luciferase reporter vector (Promega) to control for differences in transfection efficiency. Luciferase reporter gene activity was determined as described before [21]. Ratios between firefly and renilla luciferase activities were normalized to negative control co-transfections with the pM-53 and pVP16-CP vectors (Clontech) which were arbitrarily defined as 1.

In vitro transcription/translation (IVTT) and GST pull-down

IVTT reactions were carried out as described by the manufacturer (Promega) using recombined DESTination vectors in the presence of ^{35}S-Met (Hartmann Analytic, Braunschweig, Germany). For translation assays, separate IVTT reactions (1 µl), containing the pDEST17 expression vector alone, β-galactosidase expressing *lacZ* expression vector, wild-type or mutant PASKIN, were mixed with fresh reticulocyte lysate (12.5 µl) and 12.5 ng renilla luciferase cRNA (Promega). Luciferase activity was determined as above. GST-tagged proteins or GST alone were expressed in *E. coli* BL21-AI by induction with 0.2% arabinose for 4 hours and affinity purified using glutathione sepharose columns (GSTrap FF, Amersham Biosciences) by liquid chromatography (BioLogic DuoFlow, BioRad). Pull-down experiments were performed by mixing either purified proteins or 20 µl wheat germ IVTT reactions with 10 µg purified GST-tagged proteins or GST alone bound to glutathione sepharose beads.

After 30 minutes incubation at room temperature in bead binding buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.01% NP40), beads were washed 3 times with washing buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.5% NP40), boiled in sample buffer (40 mM Tris-HCl pH 6.8, 1% SDS, 50 mM β-mercaptoethanol) for 5 minutes and the proteins separated by SDS-PAGE. Gels were stained with coomassie blue, dried and radioactively labelled proteins detected by phosphorimaging (Molecular Imager FX, BioRad).

Kinase assays and mass spectrometry

His_6-PASKIN was purified from baculovirus-infected Sf9 insect cells using Ni-NTA agarose (Qiagen, Basel, Switzerland). His_6-PASKIN was incubated with 2 μg bacterially expressed and purified GST-tagged eEF1A1 (full-length or fragments) or GST alone in 25 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT for 20 minutes in the presence of 5 μCi (γ-^{33}P)ATP (Hartmann Analytic). Proteins were separated by SDS-PAGE and analyzed by phosphorimaging of the dried gels. To determine the phosphorylation site, GST-eEF1A1 was incubated with ATP and PASKIN, separated by SDS-PAGE and excised from the gel. One third of the gel band was cut into small pieces and washed twice with 100 μl 100 mM NH_4HCO_3, 50% acetonitrile and once with 50 μl acetonitrile. GST-eEF1A1 was digested in-gel with 30 μl trypsin solution (modified trypsin, Promega, 10 fg/μl in 50 mM Tris-HCl pH 8.2, 2 mM $CaCl_2$) at 37°C over night. The supernatant was removed and the gel pieces were extracted twice with 100 μl 0.1% TFA, 50% acetonitrile. All three supernatants were combined in autosampler vials, dried, and dissolved in 10 μl 0.1% formic acid for LC/ESI/MS/MS analysis run in the neutral loss mode for phosphopetides (Q-TOF Ultima API, Waters/Micromass, Manchester, UK; equipped with a capLC, Waters).

RESULTS

Generation and characterization of monoclonal antibodies against PASKIN

In order to determine the physiological sites of expression *in vivo* as well as the subcellular localization of mammalian PASKIN, monoclonal antibodies (mAbs) derived against PASKIN were generated. Therefore, a GST-PAS AB fusion protein was expressed in *E. coli*, purified and used for the immunization of mice. mABs were purified from hybridoma cell lines which reacted with GST-PAS AB but not GST alone. Two αPASKIN IgG_{2a} mAbs, mAb2 and mAb6, recognized *in vitro* transcribed and translated (IVTT) human V5-PAS AB as well as His_6-PASKIN by immunoblotting (Fig. 1A). However, none of the mAbs reacted with mouse PAS AB fragments, while αV5-tag antibodies readily detected the recombinant proteins (Fig. 1A), demonstrating that these mAbs do not cross-react with mouse PASKIN. In human testis extracts both mAbs detected a single band that co-migrated with recombinant His_6-PASKIN isolated from baculovirus-infected Sf9 cells (predicted MW: 146.4 kDa), suggesting that endogenous human PASKIN (predicted MW: 143.7 kDa) is either not or only slightly post-translationally modified in the testis (shown for αPASKIN mAb6 in Fig. 1B). No corresponding band could be observed in mouse organs, including testis and brain (Fig. 1B).

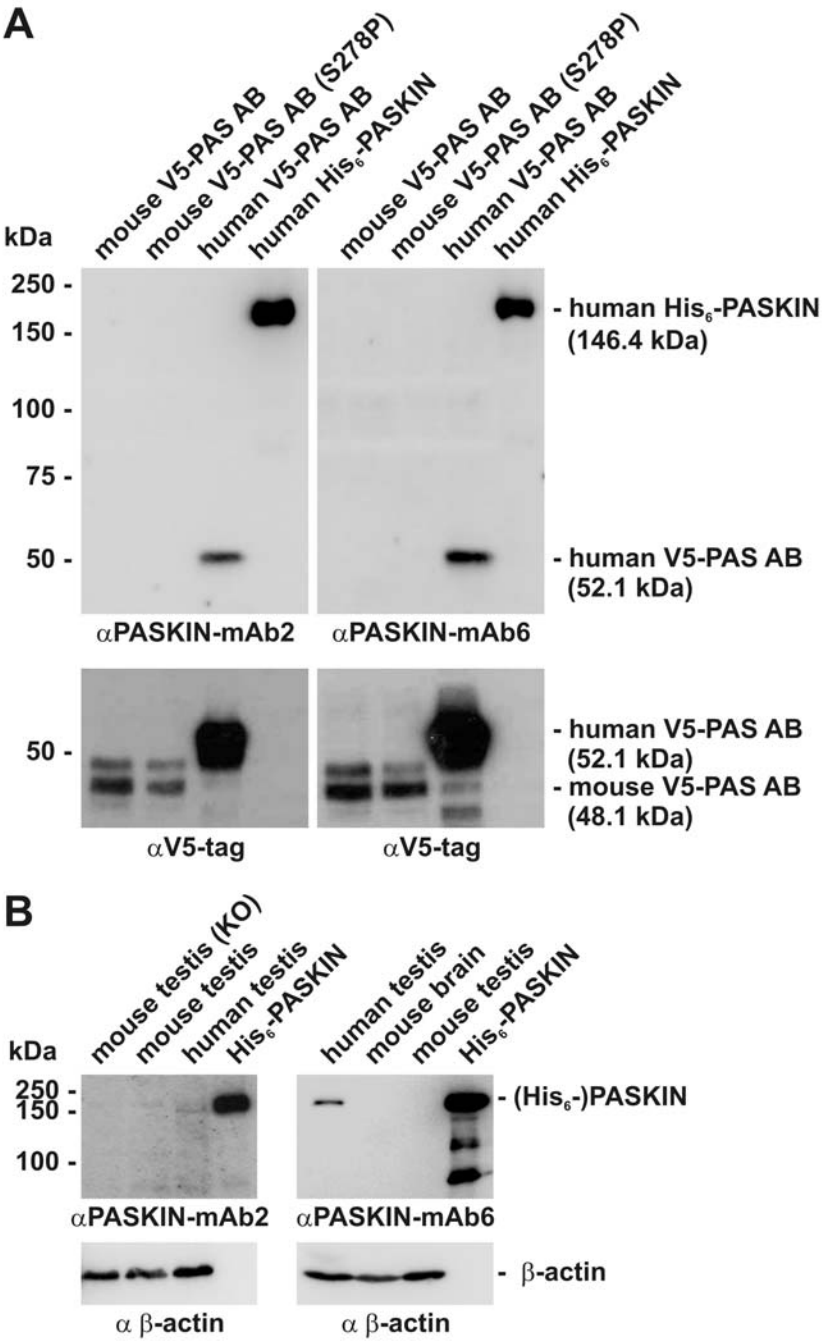

Figure 1: Generation of two monoclonal antibodies (mAbs) specific for human PASKIN. (A) Mouse monoclonal αPASKIN antibodies mAb2 and mAb6 were purified and used for immunoblot detection of human and two different clones of the mouse V5-tagged PAS AB fragments produced by IVTT and full-length human His_6-PASKIN expressed in Sf9 cells. The same blot was subsequently incubated with an anti-V5 tag antibody as control. (B) PASKIN wild-type or knock-out (KO) mouse and human tissue extracts were probed with mAb6 by immunoblotting. An extract from Sf9 cells expressing His_6-PASKIN was included as positive control. The same blot was subsequently incubated with an anti-β-actin antibody as control. Note that the αPASKIN mAbs react with human but not mouse PASKIN.

PASKIN protein localization in germ cells of the testis

We previously reported that the mouse *Paskin* gene is expressed in the testis at least 100-fold stronger than in every other organ tested [12]. As shown above, also immunoblotting revealed PASKIN protein only in the testis. Thus, αPASKIN mAb2 and mAb6 were used to analyze the PASKIN protein localization in human testis by immunohistochemistry. Signals for PASKIN were obtained in the outermost layer of cells in human seminiferous tubules, corresponding to the self-replicating spermatogonia, and in spermatocytes and round spermatids (Fig. 2A and C). At higher magnifications it became apparent that apart from some cytoplasmic staining PASKIN localized to the nuclei of spermatogonia and spermatocytes with a pattern that might match the nucleoli, at least in spermatogonia (Fig. 2B and D). In ejaculated human sperm cells, PASKIN localized mainly to the midpiece of the tail and was absent in the nucleus as determined by immunofluorescence (Fig. 2E and F).

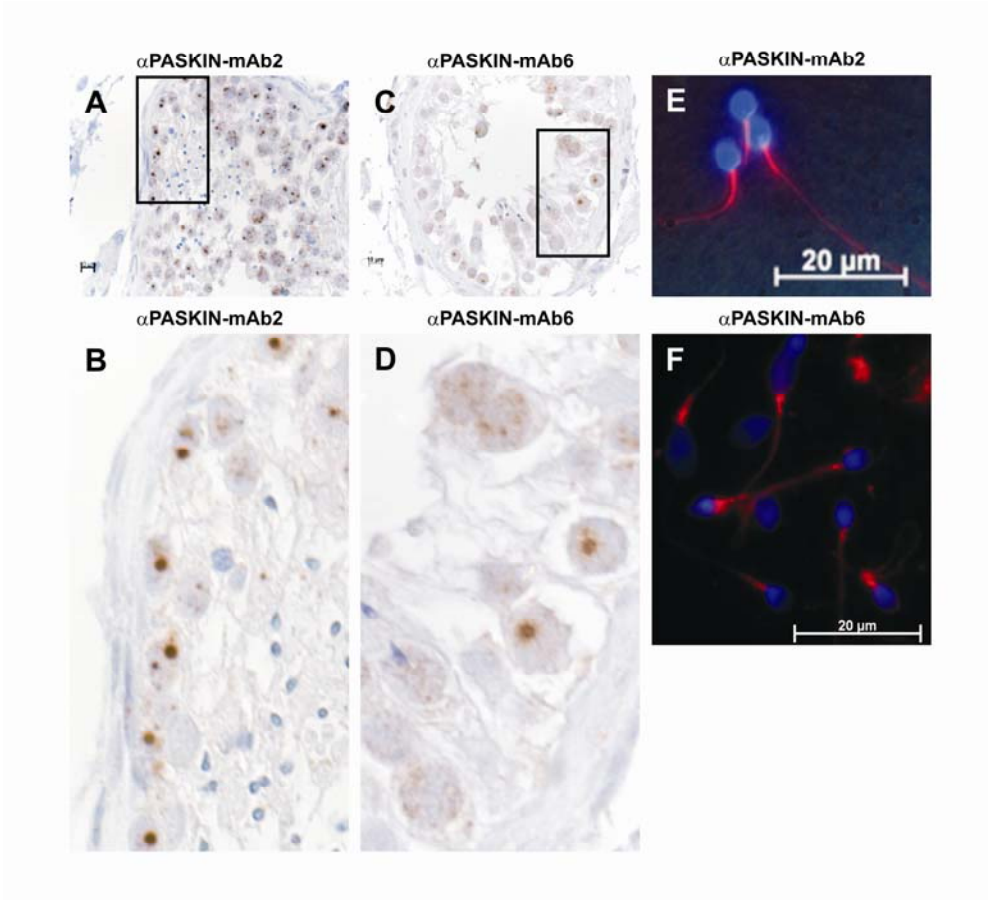

Figure 2: Subcellular localization of PASKIN in human testis and spermatozoa. Indirect immunohistochemistry of human testis (A to D) and indirect immunofluoresence of ejaculated human sperm cells (E, F). The primary αPASKIN antibodies are indicated; the secondary antibodies were coupled to HRP (A to D) or Texas red (E, F).

Cytoplasmic and nuclear localization of PASKIN in cultured cells

Ectopically overexpressed V5-PASKIN had originally been reported to localize to the cytosol of transfected HEK293 cells [6]. Likewise, we detected exogenously overexpressed human His_6-PASKIN exclusively in the cytoplasm of baculovirus-infected Sf9 insect cells by immunohistochemistry using αPASKIN mAb6 (Fig. 3A). However, by biochemical separation and immunoblotting, endogenous PASKIN had also been partially detected in the nuclear fraction of HeLa cells [6]. Moreover, a recent high-throughput screen identified PASKIN in nuclear extracts derived from HeLa cells [22]. Because PASKIN showed an unexpected nuclear pattern in human testis, we analyzed subcellular PASKIN localization in HeLa human cervical carcinoma cells as well as in NCCIT human male germ tumor cells. As shown by immunoblotting with αPASKIN mAb6, endogenous PASKIN could be detected in nuclear fractions derived from these cell lines in addition to the cytoplasmic fractions (Fig. 3B). Over-expression markedly increased the portion of PASKIN in the nuclear fractions (Fig. 3C). Confocal microscopy of HeLa cells using αPASKIN mAb6 revealed a cytoplasmic as well as a nuclear PASKIN localization, not overlaping with areas that stained weakly with Hoechst33258 DNA stain (Fig. 3D). At present it is unclear whether the nuclear speckle-like PASKIN pattern in HeLa cells corresponds to the nucleolar-like pattern in spermatogonia. The cytoplasmic PASKIN staining overlaped neither with markers for the golgi apparatus (wheat germ agglutinin and concanavalin A) nor mitochondria (mitotracker) (data not shown). Taken together, these data show that PASKIN can localize to the nucleus as well as to the cytoplasm.

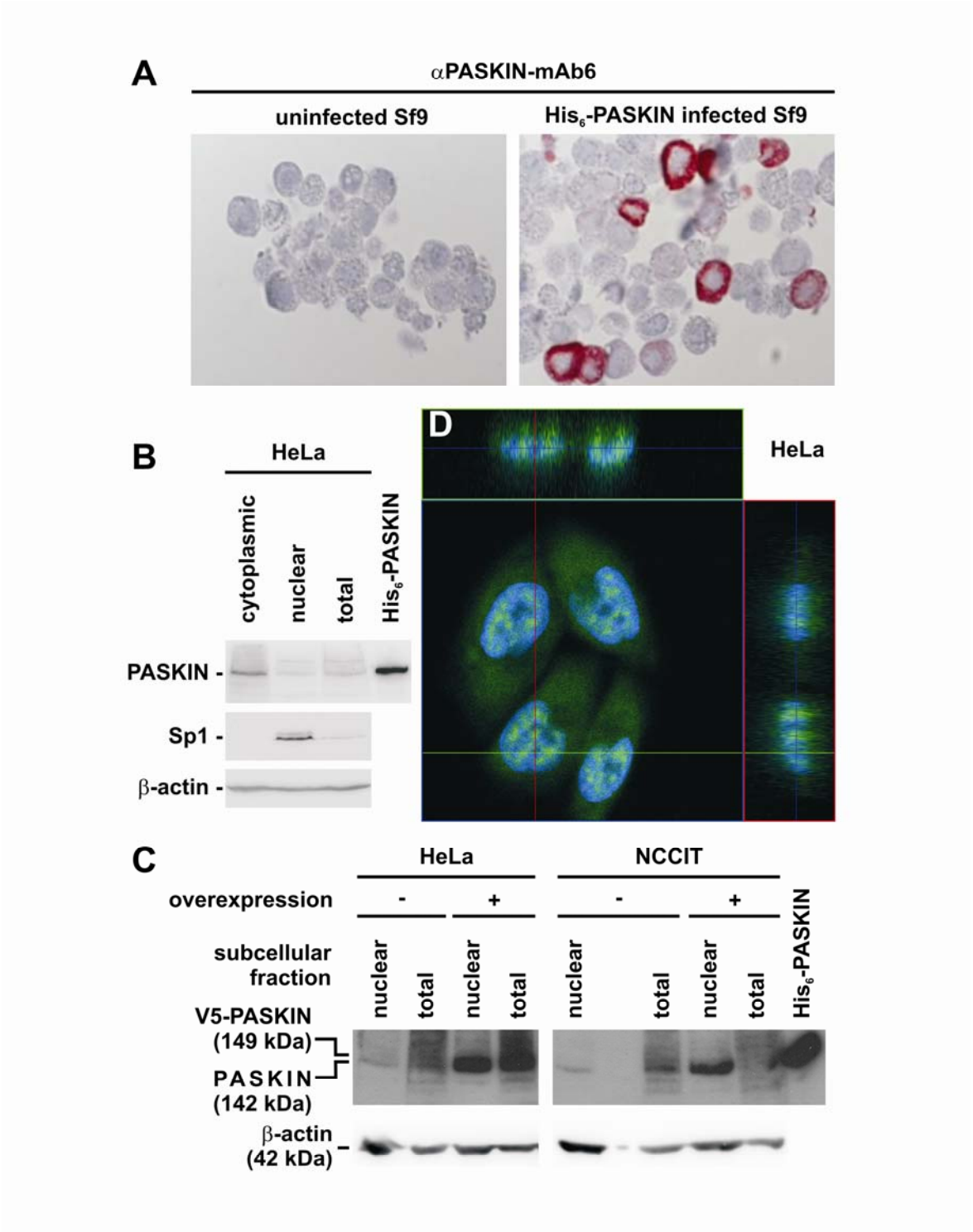

Figure 3: PASKIN localizes to the cytoplasm as well as to nuclear speckle-like structures in cultured HeLa cells. (A) Uninfected Sf9 cells, or Sf9 cells infected with human His_6-PASKIN-baculovirus for 50 hours, were fixed, pelleted, embedded in paraffin, sectioned and analyzed by immunhistochemistry using αPASKIN mAb6. (B) Immunoblotting of cytoplasmic, nuclear or total cell extracts derived from HeLa cells using αPASKIN mAb6. Subsequent incubation with antibodies derived against the transcription factor Sp1 confirmed the identity of the extracts. (C) Immunoblotting of nuclear or total cell extracts derived from untransfected HeLa and NCCIT cells, or cells transfected with a V5-PASKIN expression vector, using αPASKIN mAb6. Subsequent detection of β-actin served as control for equal loading and blotting (B, C). (D) Confocal indirect immunofluorescence microscopy of untransfected HeLa cells using αPASKIN mAb6. Nuclei were counterstained with Hoechst33258. The optical XZ and YZ planes are indicated on the sides of the XY picture to demonstrate the intranuclear localization of PASKIN.

The eukaryotic translation elongation factor eEF1A1 interacts with PASKIN

In order to better understand the function of mammalian PASKIN, a yeast two-hybrid screen for novel proteins interacting with PASKIN was performed. eEF1A1 was identified as a prey when a region spanning the PAS A and B domains was used as bait. In order to confirm this interaction, mammalian two-hybrid experiments were performed in Hep3B human hepatoma cells co-transfected with Gal4 BD-PASKIN and VP16 AD-eEF1A1 fusion constructs. The activity of a co-transfected luciferase reporter gene construct containing five Gal4 DNA-binding sites is greatly enhanced when the AD comes to lie in the vicinity of the BD. As shown in Fig. 4A, luciferase expression was significantly higher when the BD-PASKIN and AD-eEF1A1 fusion constructs were co-transfected compared with co-transfection of either the BD or the AD alone. Expression of the exogenous fusion proteins was confirmed by immunoblotting with anti-Gal4 or anti-VP16 antibodies (Fig. 4B). As shown in Fig. 4C, the KIN domain of PASKIN conferred even a stronger induction of luciferase activity than the full-length protein. Intriguingly, the two PAS domains did not stimulate luciferase expression but rather inhibited it when compared with the non-interacting negative control transfections (Fig. 4D). Thus, these results confirmed the interaction between the full-length PASKIN and eEF1A1 proteins, but showed that the PAS domains of PASKIN were inhibitory in the mammalian eEF1A1 interaction assay whereas they were activatory in the yeast eEF1A1 interaction assay.

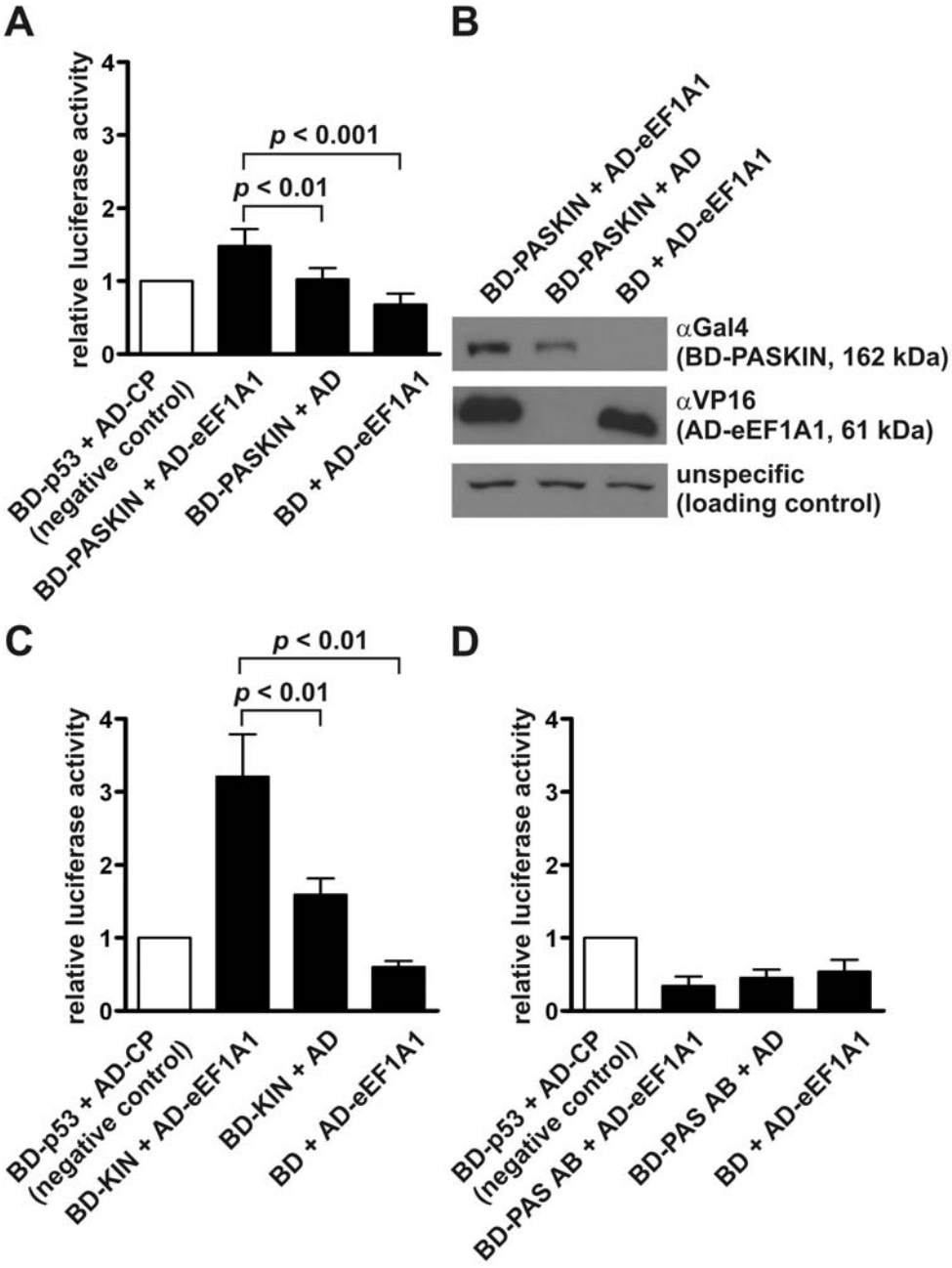

Figure 4: PASKIN:eEF1A1 interaction in Hep3B cells. Fusion proteins between VP16-AD and full-length PASKIN (A), or the KIN domain (C), or the PAS AB domain (D), together with Gal4-BD and eEF1A1 were co-transfected into Hep3B cells together with a firefly luciferase reporter gene, containing Gal4 DNA-binding sites, and a renilla luciferase control vector. Firefly luciferase reporter gene activity was determined 24 hours later, corrected for the control renilla luciferase activity and normalized to the values obtained with a negative control transfection of a non-interacting fusion protein pair. B, Immunoblotting of transfected Hep3B cells to confirm expression of the fusion proteins. Anti-Gal4 mAb detected the BD and anti-VP16 mAb detected the AD. An unspecific band reacting with the anti-Gal4 mAb served to control for equal loading. Mean values ± SEM are shown of 7 (A, C) or 3 (D) independent experiments performed in duplicates. *P* values were obtained by paired t tests and considered significant if $p < 0.05$.

Mapping of the PASKIN domains interacting with eEF1A1

The interaction between endogenous eEF1A1 and PASKIN was further confirmed by co-immunoprecipitation. Because only very low amounts of endogenous PASKIN are expressed in HeLa cells (see Fig. 3B), c-myc-tagged PASKIN was transiently overexpressed and immunoprecipitated with αPASKIN mAb6. As shown in Fig. 5A, mAb6 but not an isotype-matched control IgG co-precipitated endogenous eEF1A1.

The PASKIN:eEF1A1 interaction was then characterized by GST pull-down experiments *in vitro*. Therefore, purified full-length His_6-PASKIN was co-precipitated together with full-length, N-terminal or the C-terminal GST-tagged eEF1A1 using glutathione sepharose (Fig. 5B). Immunoblotting with αPASKIN mAb6 detected His_6-PASKIN bound to full-length and C-terminal GST-eEF1A1 but not to N-terminal GST-eEF1A1 or GST alone (Fig. 5B, top), while αGST antibodies detected all proteins on the same blot (Fig. 5B, bottom).

To map the PASKIN site(s) interacting with eEF1A1, fragments of PASKIN were transcribed and translated in wheat germ extracts and radioactively labelled by incorporation of ^{35}S-Met. These fragments were tested for interaction with purified GST-eEF1A1 fusion proteins, or GST alone, by precipitation with glutathione sepharose. In this assay, the PAS A, PAS AB (Fig. 5C) as well as the KIN (Fig. 5D) domains interacted with eEF1A1, whereas the N-terminal half of PAS A, PAS B, the N- and C-termini and the piece between the PAS and KIN domains (centerpiece) did not interact with eEF1A1.

In conclusion, both the C-terminal part of the PAS A domain as well as the KIN domain independently interact with the C-terminal part of eEF1A1, providing an explanation why eEF1A1 could interact with the PAS domain of PASKIN in yeast and with the KIN domain of PASKIN in Hep3B cells.

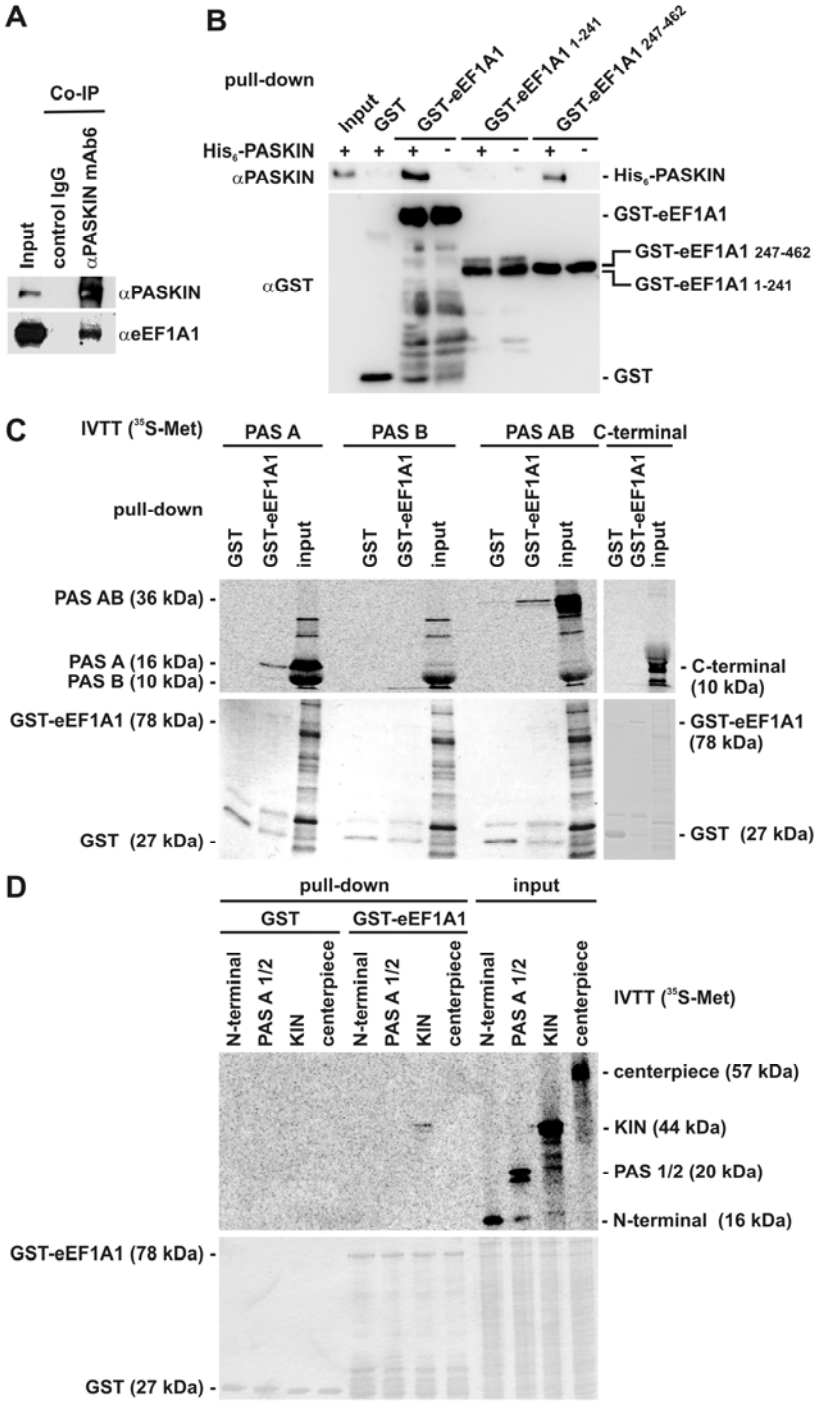

Figure 5: PASKIN:eEF1A1 protein-protein interaction. (A) Co-immunoprecipitation of endogenous eEF1A1 with c-myc-tagged PASKIN expressed in HeLa cells. αPASKIN mAb6 but not control antibodies co-precipitated eEF1A1 as shown by immunoblotting. (B) Purified recombinant proteins derived from *E. coli* (GST alone or GST-tagged eEF1A1, full-length or the indicated fragments) or Sf9 insect cells (His_6-PASKIN) were mixed, incubated and analyzed by GST pull-down with glutathione-sepharose followed by immunoblotting using αPASKIN and αGST mAbs. (C, D) Protein:protein interaction between radioactively labelled fragments of PASKIN produced by IVTT and purified recombinant GST-eEF1A1 fusion protein, or GST alone, were analyzed by GST pull-down with glutathione-sepharose followed by SDS-PAGE and phosphorimaging (upper panels in C and D). "Input" reflects fractions of the IVTT reactions before GST pull-down. The coomassie-stained gels are shown in the lower panels of (C) and (D) to demonstrate equal pull-down efficiency. Note that the full-length GST-eEF1A1 fusion protein is barely visible in (C).

eEF1A1 is phosphorylated by PASKIN at Thr432

We next determined whether eEF1A1 can be phosphorylated by PASKIN. Therefore, His_6-PASKIN was purified from baculovirus-infected Sf9 insect cells. As expected from published results [6], His_6-PASKIN auto-phosphorylated in the presence of ^{33}P-ATP, demonstrating that it was functionally active (Fig. 6A). PASKIN also phosphorylated full-length GST-eEF1A1 and GST-eEF1A1$_{247-462}$, whereas GST-eEF1A1$_{1-241}$ was phosphorylated to a clearly lower extent (Fig. 6A) and GST alone was not phosphorylated at all (data not shown). As shown by coomassie staining, all recombinant GST-tagged proteins were present in approx. equimolar concentrations (Fig. 6B), whereas the concentrations of the kinases were too low to be detected by this technique.

To determine the phosphoacceptor site of eEF1A1, full-length GST-eEF1A1 was phosphorylated by His_6-PASKIN, separated by SDS-PAGE and in-gel digested with trypsin. Separation of the fragments by liquid chromatography followed by electrospray ionization-tandem mass spectrometry in the neutral loss mode for phosphopetides identified the C-terminal peptide DMRQTVAVGVIK with Thr432 as the only phosphoacceptor site in this analysis (Fig. 6C).

To confirm the role of Thr432 it was mutated to alanine by site-directed mutagenesis. As shown in Fig. 6A, phosphorylation of full-length GST-eEF1A1T432A by His_6-PASKIN was almost completely abolished. However, while GST-eEF1A1$_{247-462}$T432A also showed impaired phosphorylation by His_6-PASKIN, some residual phosphorylation of this eEF1A1 fragment was still detectable, suggesting that T432A is the major but not the only phosphoacceptor site on eEF1A1.

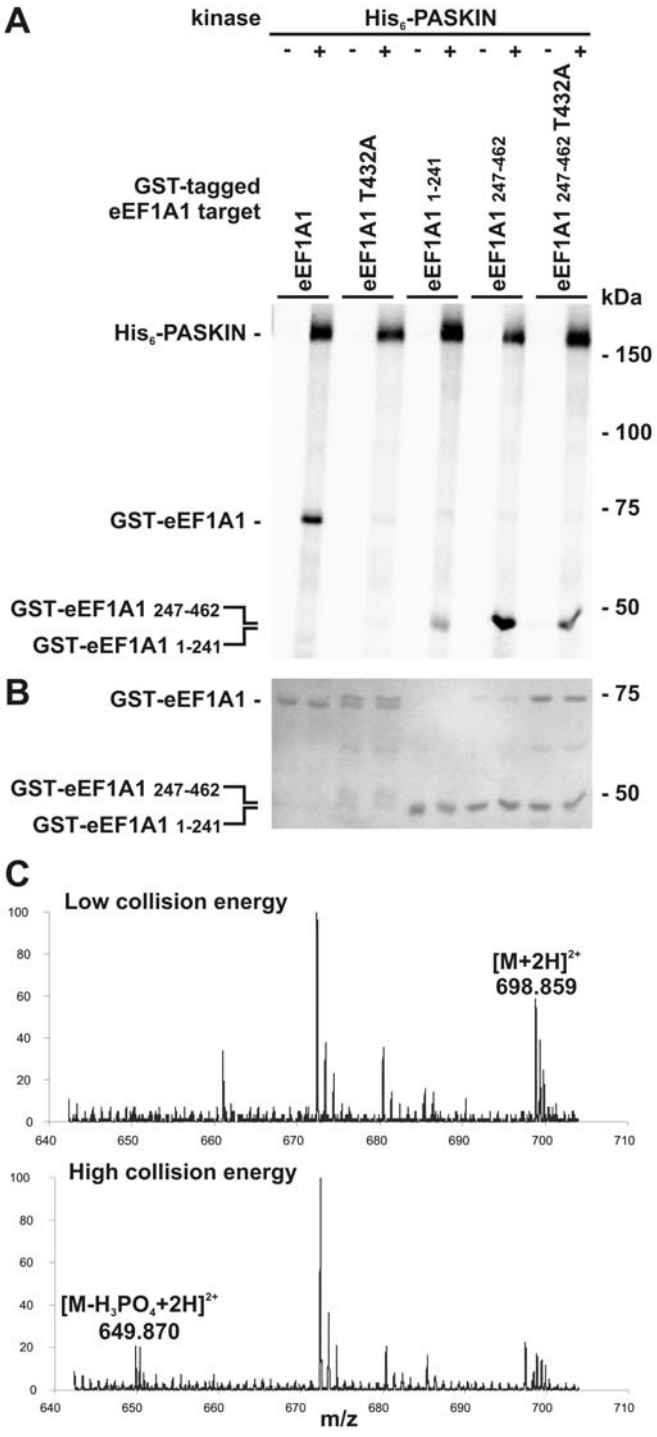

Figure 6: PASKIN auto-phosphorylation and eEF1A1 target-phosphorylation. (A) Purified recombinant GST-eEF1A1 was *in vitro* phosphorylated by His_6-PASKIN, separated by SDS-PAGE and detected by phosphorimaging. T432A mutants and fragments of eEF1A1 were included as indicated. (B) Corresponding coomassie-stained gel to indicate equimolar loading of phosphorylation target proteins. (C) Neutral loss measurement by LC/ESI/MS/MS of phospho-eEF1A1 following in-gel digestion. The mass difference of 48.989 between the measurements of the peptide DMRQTVAVGVIK at low collision energy (upper trace) and at high collision energy (lower trace) shows the presence of a phosphorylation site in this peptide. Thr432 was identified as the main PASKIN-dependent phosphorylation site in eEF1A1.

PASKIN increases translation efficiency in a cell-free in vitro assay

PASKIN-dependent phosphorylation of eEF1A1 suggests that PASKIN might influence translation efficiency. To test for this hypothesis, wild-type and kinase-inactive mutants of PASKIN were synthesized by IVTT in rabbit reticulocyte lysates (Fig. 7A). PASKIN was then tested for its effects on translation of a renilla luciferase cRNA in fresh rabbit reticulocyte lysates containing endogenous eEF1A1 (see Fig. 8A). While the empty IVTT expression vector, β-galactosidase, and kinase-inactive PASKIN T1161A and/or T1165A mutants [6] did not significantly alter protein synthesis, wild-type PASKIN increased renilla luciferase protein synthesis by 80% (Fig. 7B).

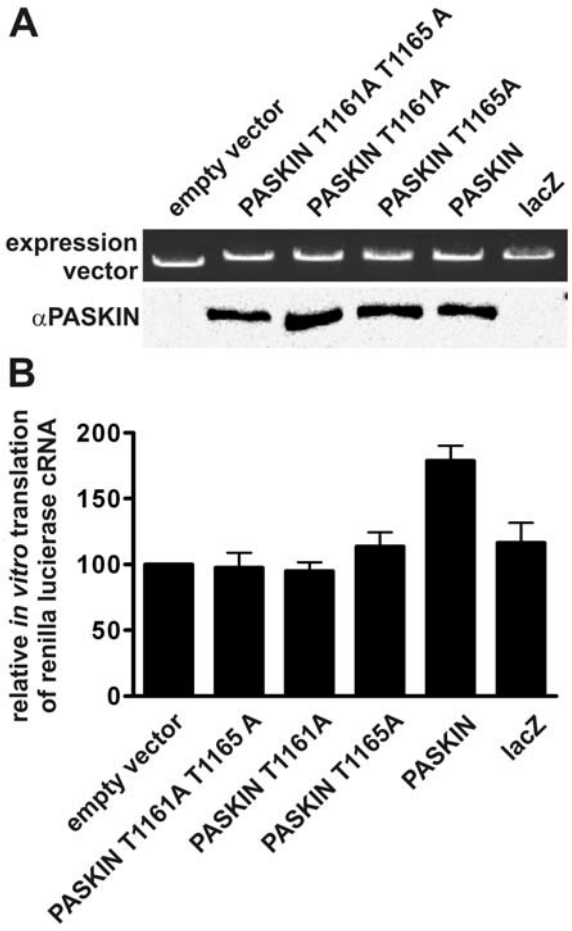

Figure 7: PASKIN increases translation in a cell-free translation assay. (A) Wild-type PASKIN or the indicated kinase-inactive PASKIN mutants were produced by IVTT. Linearized expression vectors (agarose gel electrophoresis and ethidium bromide staining; top panel) were used to produce similar amounts of PASKIN protein (immunoblotting using αPASKIN mAb6; bottom panel). (B) Translation was assayed by adding fractions of these IVTT reactions to reticulocyte lysates containing renilla luciferase cRNA. Luciferase activities were normalized to the empty vector control and are shown as mean values ± SEM of n = 3 independent experiments. IVTT reactions using a *lacZ* vector expressing β-galactosidase served as negative controls.

Co-localization of PASKIN and eEF1A1 in the midpiece of the sperm tail

Functional interaction between PASKIN and eEF1A1 requires a cellular co-localization. Thus, we next analyzed the subcellular localization of eEF1A1. Therefore, HeLa cells were analyzed by immunofluorescence using an αeEF1A1 mAb. Immunoblotting of IVTT reticulocyte extracts, containing or not containing an eEF1A1 expression vector, and of HeLa total cell extracts demonstrated the presence of eEF1A1 in HeLa cells and confirmed the specificity of the antibody (Fig. 8A). Indirect immunofluorescence with this antibody followed by an Alexa488-coupled secondary antibody localized eEF1A1 exclusively to the cytoplasm (Fig. 8B). A similar cytoplasmic localization was observed in germ cells of the human testis by immunohistochemistry (Fig. 8C). Interestingly, by indirect immunofluorescence using a Texas red-coupled secondary antibody eEF1A1 was also detected in the tail of ejaculated human sperm (Fig. 8D). Confocal immunofluorescence microscopy revealed a very similar pattern of eEF1A1 and PASKIN, suggesting co-localization of the two proteins in the midpiece of the sperm tail (Fig. 8E).

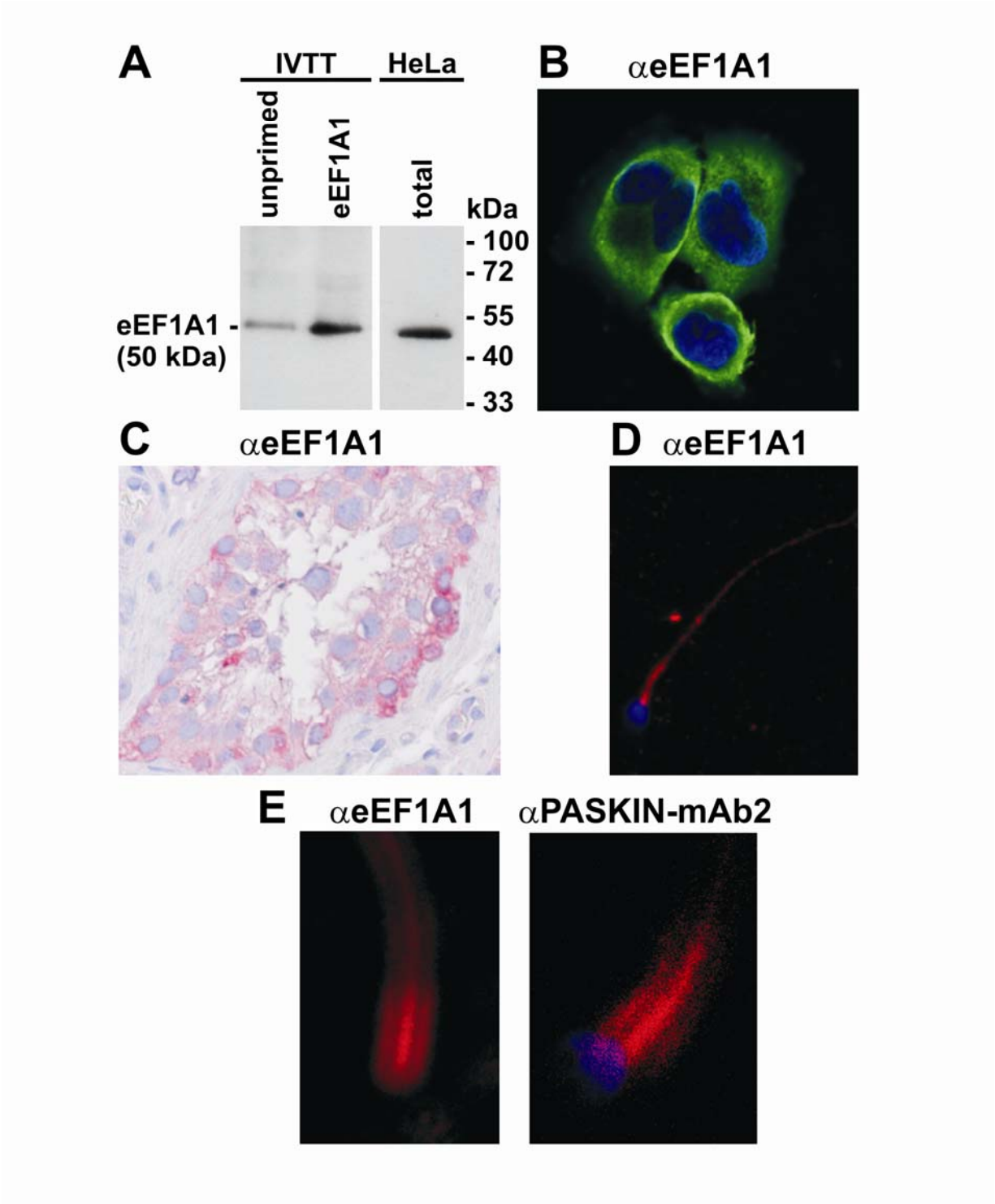

Figure 8: eEF1A1 and PASKIN co-localization in HeLa and sperm cells. (A) Immunoblot analysis of eEF1A1 in unprimed reticulocyte lysates, or following IVTT of an eEF1A1 expression vector, and in untransfected HeLa cells, demonstrating the specificity of the αeEF1A1 mAb. (B) Confocal indirect immunofluorescence microscopy of untransfected HeLa cells using αeEF1A1 primary and Alexa488-coupled secondary antibodies. (C) Immunohistochemistry of human testis using αeEF1A1 mAb (red). Note that both in HeLa (B) as well as in testis (C), eEF1A1 localizes to the cytoplasm, suggesting that this is the main site of interaction with PASKIN in these cells. (D) Indirect eEF1A1 immunofluorescence microscopy of human ejaculated sperm using αeEF1A1 primary and Texas red-coupled secondary antibodies. E, Confocal indirect immunofluorescence microscopy of the midpiece of the human sperm tail using αeEF1A1 or αPASKIN mAb6 primary and Texas red-coupled secondary antibodies. Nuclei were counterstained with DAPI (B) or Hoechst33258 (D, E).

DISCUSSION

We previously identified PASKIN as a gene that is ubiquitously expressed at low abundance in most mouse organs analyzed. In contrast, much higher mRNA levels were found in the testis [5,12]. Presumably, these low levels in somatic tissues can further be induced by changing environmental conditions, as suggested by the recent demonstration that high glucose induces PASKIN in pancreatic β-cells [10]. Of note, in a large-scale characterization of nuclear phosphoproteins, a PASKIN-derived phosphopeptide (Ser116) was identified in HeLa cells, suggesting a potentially regulatable function for PASKIN [22]. Thus, HeLa appeared to be a valuable tool to identify PASKIN downstream targets in non-germline cells, and we identified the translation elongation factor eEF1A1 as a novel PASKIN interaction partner by yeast two-hybrid screening.

We further demonstrated that the C-terminal part of eEF1A1 interacts with both the PAS A and KIN domains of PASKIN as schematically summarized in Fig. 9.

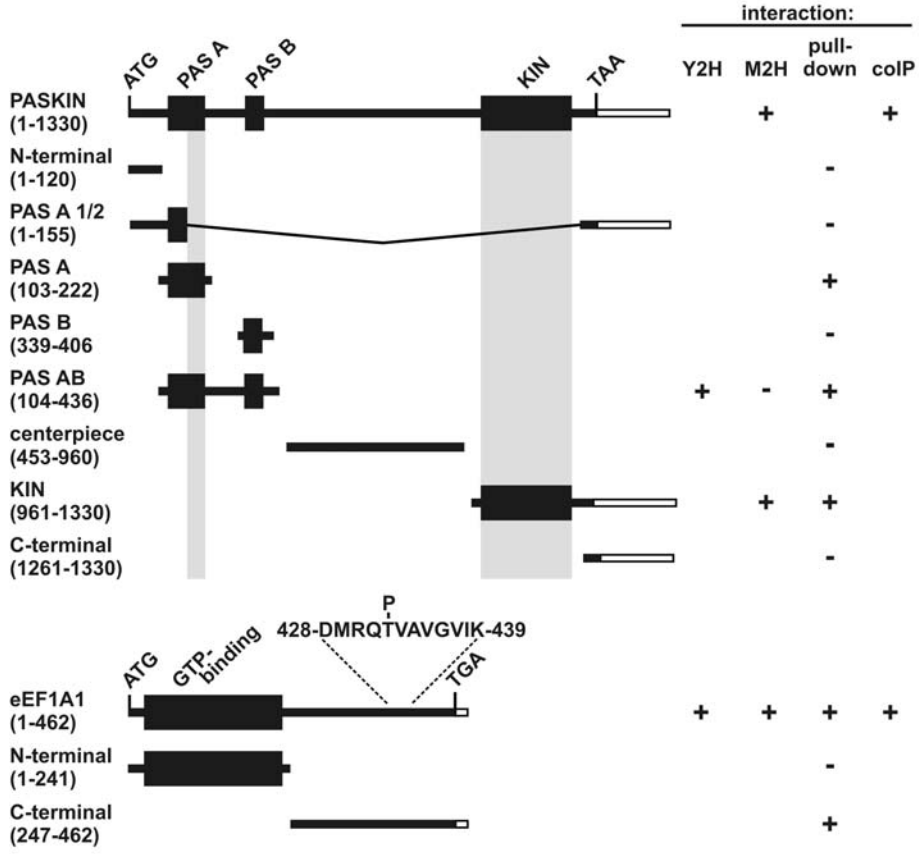

Figure 9: Scheme of the PASKIN:eEF1A1 interaction. Results from Figs. 4 and 5 are summarized on the right part of the picture. The eEF1A1 phosphopeptide identified following PASKIN-dependent phosphorylation is indicated. Y2H, yeast two-hybrid; M2H, mammalian two-hybrid; coIP, co-immunoprecipitation.

Human eEF1A1 is phosphorylated by PASKIN at Thr432, suggesting that PASKIN regulates eEF1A1 function. Several other kinases are known to phosphorylate eEF1A1, including PKCδ, Rho-associated kinase, and S6 kinase [23-25]. Interestingly, PKCδ has been shown previously to phosphorylate the corresponding Thr431 PASKIN target site in mouse eEF1A1 [23], and we found PKCδ-dependent human eEF1A1 phosphorylation at Thr432 (data not shown). Up to date the functional consequences of this phosphorylation remain unknown. Insulin stimulation of protein synthesis involves S6 kinase-dependent eEF1A1 phosphorylation [25], and here we could demonstrate that PASKIN increases protein translation in a cell-free translation assay. Thus, PASKIN-dependent phosphorylation of eEF1A1 might link energy metabolism with protein translation in mammalian cells as it has been demonstrated previously for yeast [8]. However, the major site of PASKIN expression *in vivo* is the testis. Because no corresponding cell culture model is available, at present we can only speculate on a role for PASKIN in protein translation in male germ cells.

In addition to protein translation, PASKIN-dependent eEF1A1 phosphorylation might also have completely different consequences. In fact, eEF1A1 displays a multitude of functions unrelated to protein synthesis, including cytoskeletal organization, signal transduction, RNA synthesis, proteasomal degradation of damaged proteins, apoptosis and activation of the heat-shock transcription factor; eEF1A1 hence is involved in major diseases such as diabetes and cancer [15,17,26]. We thus reasoned that the identification of the subcellular compartments which show co-localization of eEF1A1 and PASKIN might give further hints to the physiological functions of PASKIN-dependent eEF1A1 phosphorylation. In baculovirus-infected insect cells, we found PASKIN exclusively in the cytoplasm, consistent with a previous report on transiently transfected HEK293 cells [6]. Cytoplasmic localization of endogenous PASKIN was also found in HeLa cells, suggesting a role for the PASKIN:eEF1A1 interaction in this compartment. However, in order to study the major physiological site of PASKIN expression, the testis, it was necessary to generate specific αPASKIN antibodies. Unexpectedly, endogenous PASKIN showed an apparently nucleolar pattern in spermatogonia and a more speckled nuclear pattern in spermatocytes and in HeLa cells in addition to the cytoplasmic localization. A previously not recognized phosphorylation site of PASKIN (Ser116) has been identified in nuclear extracts derived from HeLa cells [22]. It is currently unknown whether phosphorylation of PASKIN Ser116 affects PASKIN subcellular localization, PASKIN kinase activity, or both.

Although also eEF1A1 has been reported to localize to the nucleus under certain circumstances [15], we detected endogenous eEF1A1 exclusively in the cytoplasm of HeLa

cells and germ cells of human testis. However, there was a complete, and thus far not recognized, overlap in PASKIN and eEF1A1 expression and localization in mature human spermatozoa. Because these cells ceased protein translation, a translation-unrelated function of PASKIN-dependent eEF1A1 phosphorylation appears to be likely. The midpiece of the sperm tail is a highly organized structure comprising cytoskeletal components, the mitochondria, and most of the remaining cytoplasmic liquid which contains several testis-specific isoforms of glycolytic enzymes as well as of the transcription factor HIF-1α, another PAS domain protein [13,27,28]. Thus, this site is involved in the response to external stimuli by regulation of energy flux, heat-stress response and apoptosis, features that would be consistent with the known functions of PASKIN and eEF1A1. Future experiments will be required to identify the external stimuli affecting these features.

ACKNOWLEDGEMENTS

The authors wish to thank R. Fischer for the generation of monoclonal antibodies; J. Rutter, J. Nesper, S. Hägele and S. Schweyer for the generous gifts of plasmids and cells; U. Ziegler and J. Rohrer for helpful advice and discussions; and A.-K. Hellberg, B. Stier and C. Franke for excellent technical assistance. This work was supported by grants from the BMBF (NBL3 to D.M.K.), the DFG (Ka 1269/7-1 to D.M.K.), the Forschungsschwerpunkt Reproduktionsmedizin of the University of Lübeck (to K.F.W.), the Hartmann-Müller Stiftung (to R.H.W.) and the SNF (3100A0-104219 to R.H.W. and G.C.).

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6. PHOSPHOLIPIDS ACTIVATE PASKIN AUTOPHOSPHORYLATION

Phospholipids stimulate the autophosphorylation of the PAS domain kinase PASKIN

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Key words: energy homeostasis, glycogen synthesis, insulin secretion, nitrogen fixation, phospholipase, PAS domain, protein translation, testis.

ABSTRACT

The PAS domain serine/threonine kinase PASKIN, or PAS kinase, links energy flux and protein synthesis in yeast and regulates glycogen synthesis and protein translation in mammals. According to the current model, binding of a putative ligand to the PAS domain disinhibits the kinase domain, leading to PASKIN autophosphorylation and full kinase activity. Synthetic ligands have been reported to be bound by PASKIN. However, it was unknown whether endogenous ligands exist and how they affect PASKIN kinase activity. Here, we identified phospholipids as potent stimulators of PASKIN autophosphorylation. Immobilized phospholipids dose-dependently bound purified PASKIN protein. Phospholipase C but not D abrogated phospholipid interactions with PASKIN, suggesting that phosphatidic acid is the minimal moiety binding to PASKIN. Indeed, phosphatidic acid was sufficient to fully activate PASKIN autophosphorylation. Interestingly, while phospholipids stimulated PASKIN autophosphorylation, phosphorylation of the eukaryotic translation elongation factor eEF1A1, a recently identified PASKIN target protein, was inhibited.

INTRODUCTION

In lower organisms, the PAS (Per-Arnt-Sim) domain is often found in environmental protein sensors involved in the perception of light intensity, oxygen partial pressure, redox potentials, voltage and certain ligands [1]. In mammals, the PAS domain is mainly found as a heterodimerization interface of transcription factors involved in dioxin signalling, the circadian clock and oxygen sensing [2-4]. We and others previously identified a novel mammalian PAS protein, alternatively called PASKIN [5] or PAS kinase [6]. PASKIN contains two PAS domains (PAS A and PAS B) and a serine/threonine kinase domain which might be regulated in *cis* by binding of so far unknown ligands to the PAS domain [7]. PASKIN shows a striking structural similarity to the bacterial oxygen sensor FixL that contains an oxygen-binding heme group within its PAS domain [5]. Following derepression by ligand binding, autophosphorylation in *trans* results in the "switch-on" of the kinase domain of FixL and probably of PASKIN as well [6]. PSK1 and PSK2, the budding yeast homologs of PASKIN, phosphorylate three translation factors and two enzymes involved in the regulation of glycogen and trehalose synthesis, thereby coordinately controlling translation and sugar flux [8]. In mammals, PASKIN-dependent phosphorylation inhibits the activity of glycogen synthase [9]. PASKIN has also been suggested to be required for glucose-dependent transcriptional induction of preproinsulin gene expression, which might be related to PASKIN-dependent regulation of the nuclear import of pancreatic duodenal homeobox-1 transcription factor PDX-1 [10,11]. However, by using normally fed PASKIN-deficient knock-out mice, we could not observe any PASKIN-dependent difference in insulin gene expression or in glucose tolerance [12] [13].

We previously found that the eukaryotic translational elongation factor eEF1A1 is phosphorylated by PASKIN at threonine 432, probably leading to increased translation elongation [14]. Because eEF1A1 is also phosphorylated by PKCδ, we compared the features of the two kinases, in particular their activation by lipids. As expected, PKCδ kinase activity could be induced by a mixture of phosphatidylserine and diacylglycerol. Surprisingly, PASKIN kinase activity could also be stimulated by phospholipids but not by diacylglycerol.

MATERIALS AND METHODS

Plasmids

All cloning work was carried out using Gateway technology (Invitrogen, Basel, Switzerland). Human PASKIN cDNA (kindly provided by J. Rutter, Salt Lake City, UT, USA) was cloned into pENTR (Invitrogen) as described elsewhere [14]. To generate expression vectors for fusion proteins, ENTRy vectors were recombined *in vitro* with DESTination vectors using LR Clonase recombination enzyme mix (Invitrogen). pDEST15, pDEST17 and pMAL were used to generate bacterial expression vectors for glutatione-S-transferase (GST)-, His_6- and maltose-binding protein (MBP)-tagged fusion proteins, respectively. pDEST10 and pDEST20 were used to generate expression vectors for His_6- and GST-tagged fusion proteins, respectively, in the baculovirus/Sf9 insect cell system (Invitrogen). pcDNA3.1/nV5-DEST was used to express N-terminal V5-tagged proteins in mammalian cells or in rabbit reticulocyte lysates.

PASKIN expression and purification

His_6-PASKIN was purified from baculovirus-infected Sf9 insect cells using passive lysis buffer (Promega, Madison, WI) and Ni^{2+}-NTA agarose (Qiagen, Basel, Switzerland). His_6-PASKIN purity was checked by SDS-PAGE followed by coomassie or SYPRO ruby staining (Molecular Probes/Invitrogen), or by immunoblotting using monoclonal anti-PASKIN antibody mAb6 [14]. PASKIN purity was quantified by fluorescence imaging using a sensitive CCD camera (FluorChem8900, AlphaInnotech, Witec, Littau, Switzerland).

Kinase assays

His_6-PASKIN or PKCδ (Invitrogen) was incubated with or without 2 μg bacterially expressed and purified GST-tagged or MBP-tagged target proteins in kinase buffer (25 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) for 20 minutes in the presence of 3 μCi (γ-^{33}P)ATP (Hartmann Analytic, Braunschweig, Germany). Proteins were separated by SDS-PAGE and analyzed by phosphorimaging of the dried gels (Molecular Imager FX, BioRad, Reinach, Switzerland). Lipids were dissolved in $CHCl_3$, aliquotted in test tubes and the $CHCl_3$ evaporated under a stream of nitrogen. Lipids were then resuspended in kinase assay master mixes by thoroughly vortexing.

Lipid binding assays

Interactions between PASKIN and lipids were measured by an ELISA-based assay described previously [15]. Briefly, 96-well plates (Sarstedt, Nümbrecht, Germany) were coated overnight with phospholipids dissolved in methanol, followed by blocking with 3% BSA in PBS for 1 hour. Purified His_6-PASKIN (100 ng) was diluted in kinase buffer and allowed to bind for 1 hour at 30°C. After three washing steps (0.3% Tween-20 in PBS), bound PASKIN was detected by anti-PASKIN mAb6, followed by secondary goat anti-mouse HRP-conjugated antibodies (Pierce, Perbio, Lausanne, Switzerland) using the 3,3',5,5'-tetramethylbenzidine substrate kit (Pierce). The peroxidase reaction was stopped by adding H_2SO_4 (1 M final concentration) and absorbance was determined at 450 nm in a microplate reader. For phospholipase (PL) experiments, phospholipid-coated 96-well plates were treated with 0.2 units PLC or PLD (Sigma), diluted in reaction buffer (120 mM $CaCl_2$, 300 mM sodium acetate pH 5.6) for 1 hour at room temperature.

RESULTS

Autophosphorylation of recombinant PASKIN is activated by lipids

Functionally active His_6-tagged PASKIN was affinity-purified from baculovirus-infected Sf9 insect cells (Fig. 1A). Following staining with a fluorescent dye, quantification of the 146.4 kDa band by fluorimaging of SDS-PAGE gels revealed a purity of at least 80% (Fig. 1B). By immunoblotting, many of the smaller bands represent degradation products as they reacted with a monoclonal anti-PASKIN antibody, suggesting an even higher proportion of PASKIN in this preparation (Fig. 1C).

As reported previously [6], recombinant His_6-PASKIN autophosphorylated in the prescence of radioactively labelled ATP (Fig. 1D) and a T1165A mutant form of PASKIN completely lacked autophosphorylation activity (data not shown). To investigate whether PASKIN autophosphorylation within its kinase domain (Thr1161 and Thr1165) occurs *in cis* or *in trans*, the PAS domain (GST-PAS AB) or the kinase domain (MBP-KIN) of PASKIN, or the GST or MBP tags alone, were added to the PASKIN kinase reactions. As expected, the PAS domain or the tags alone were neither phosphorylated by PASKIN nor affected PASKIN autophosphorylation (Fig. 1D). However, rather than being phosphorylated by PASKIN, the presence of the PASKIN kinase domain inhibited PASKIN autophosphorylation (Fig. 1D).

PASKIN and PKCδ are both known to autophosphorylate [6,16], as well as to phosphorylate the eukaryotic translation elongation factor eEF1A1 [14]. Because PKCδ kinase activity is known to be stimulated by diacylglycerol (DAG) and phosphatidylserine (PS) [17], we wondered whether additional similarities exist between PASKIN and PKCδ. Interestingly, a mixture of PS and DAG (used in the form of dioctanoylglycerol, DOG) not only enhanced PKCδ but also PASKIN autophosphorylation (Fig. 1E).

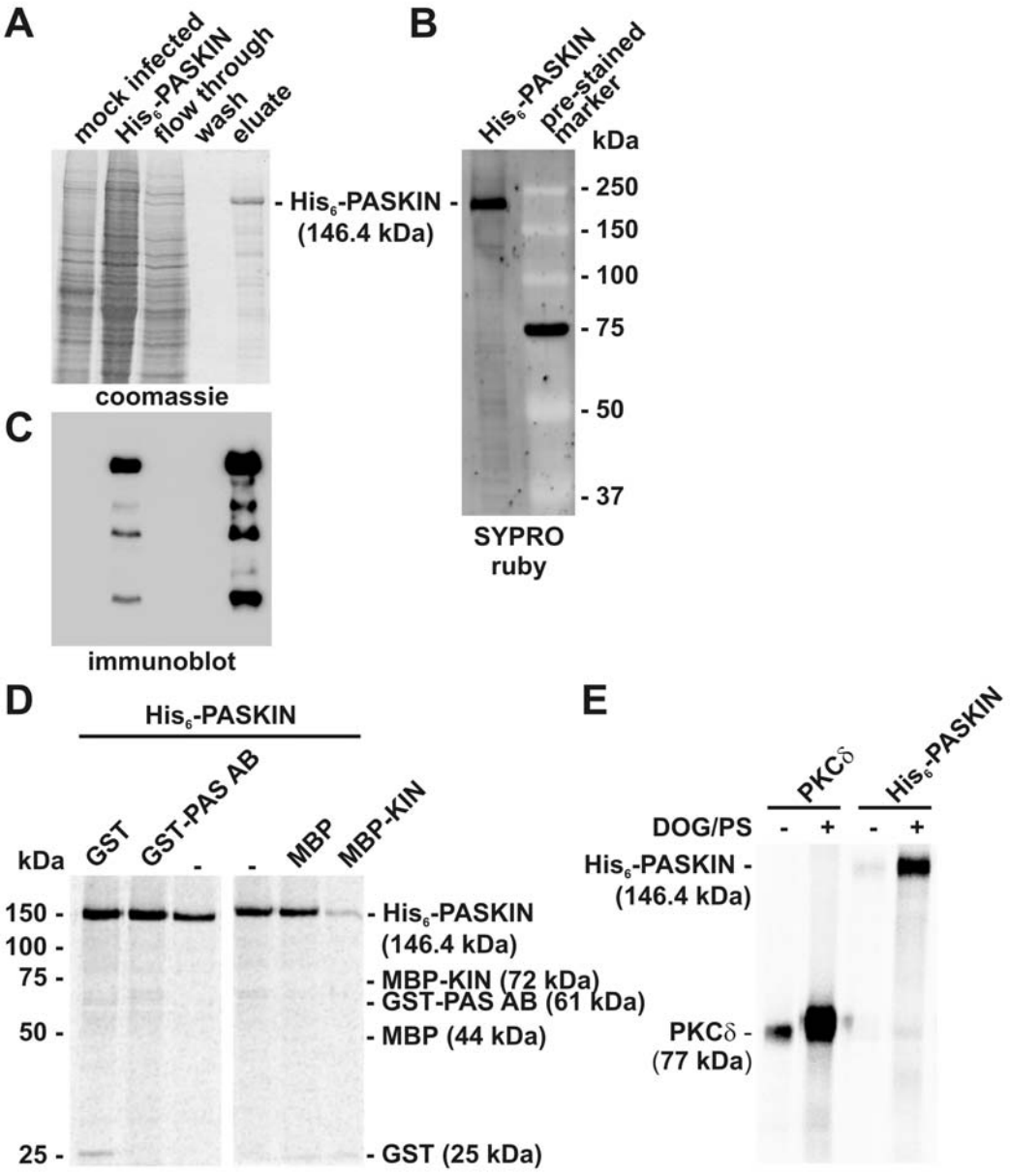

Figure 1: Purification and autophosphorylation of PASKIN. A, Coomassie-stained SDS-PAGE gel of Sf9 cell extracts infected with baculovirus containing His_6-PASKIN expression vectors. PASKIN was purified from these extracts by Ni^{2+}-NTA agarose affinity chromatography as indicated. B, PASKIN was separated by SDS-PAGE and stained with a fluorescent dye to determine its purity. C, Immunoblotting of the fractions shown in A, using an anti-PASKIN monoclonal antibody. D, PASKIN autophosphorylation in the presence or absence of GST, GST-PAS AB, MBP, or MBP-KIN. E, A DOG/PS lipid mixture stimulates PKCδ as well as PASKIN autophosporylation. D and E, Kinase reaction mixtures were separated by SDS-PAGE and the radioactive bands of the dried gels were detected by phosphorimaging.

Phospholipids but not DAG stimulate PASKIN autophosphorylation

In order to systematically analyze the lipid activation of PASKIN, all major phospholipids were compared for their effects on PASKIN and PKCδ autophosphorylation. As shown in Fig. 2, all tested phospholipids but not DOG alone increased PASKIN autophosphorylation. In contrast, PKCδ autophosphorylation was induced by DOG alone, to some extent also by PS or phosphatidylcholine (PC), but all other phospholipids had only marginal effects on PKCδ. As known before, a mixture between DOG and PS was required to maximally induce PKCδ activity. However, combining DOG with phospholipids did not further induce PASKIN (data not shown).

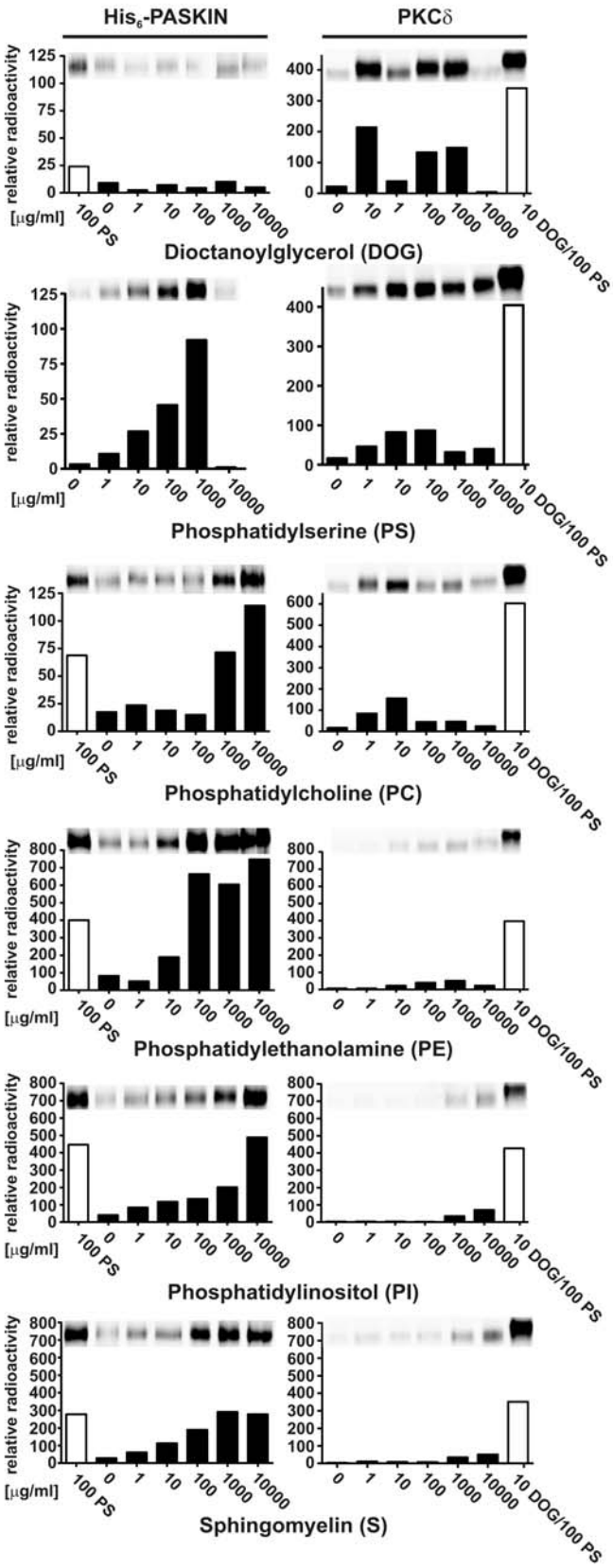

Figure 2: PASKIN autophosphorylation is stimulated by various phospholipids. PASKIN or PKCδ autophosphorylation was stimulated with increasing amounts of the indicated (phospho-)lipids and quantified by phosphorimaging after SDS-PAGE.

PASKIN binds a distinct set of phospholipids

To characterize phospholipid binding by PASKIN, an ELISA-based assay was applied. Therefore, 96-well plates were coated with increasing amounts of a variety of phospholipids and remaining binding sites were blocked with BSA. Purified recombinant PASKIN was allowed to bind to the phospholipids and the amount of bound PASKIN was determined by a monoclonal anti-PASKIN antibody. As shown in Fig. 3A, PASKIN bound to the phospholipids phosphatidylethanolamine (PE), PS and phosphatidic acid (PA), but not to DOG or to the other phospholipids (PC; phosphatidylinositol, PI; sphingomyelin, S) tested. This was somewhat unexpected since PASKIN autophosphorylation was also induced by other phospholipids. However, when higher amounts of PASKIN were used in this assay, binding to PC could also be demonstrated (Fig. 3B), suggesting different PASKIN affinities for the various phospholipids.

The finding that PA but not DOG strongly bound PASKIN suggested that PLD might target PASKIN by converting phospholipids into PA. To directly demonstrate this assumption, constant amounts of bound PC (1 μg) were treated with PLD or PLC followed by binding to increasing amounts of PASKIN. As shown in Fig. 3B, PLD but not PLC converted PC into a lipid binding PASKIN efficiently. On the other hand, the lipid affinity of a constant amount of PASKIN (100 ng) was enhanced when increasing amounts of PC were treated with PLD, but not with PLC, (Fig. 3C). In summary, PLD-dependent PA production, but not PLC-dependent DOG production, increased the strength of the interaction between PASKIN and phospholipids. We could not detect higher PASKIN affinities for any of the phospholipids tested than for the PA backbone alone.

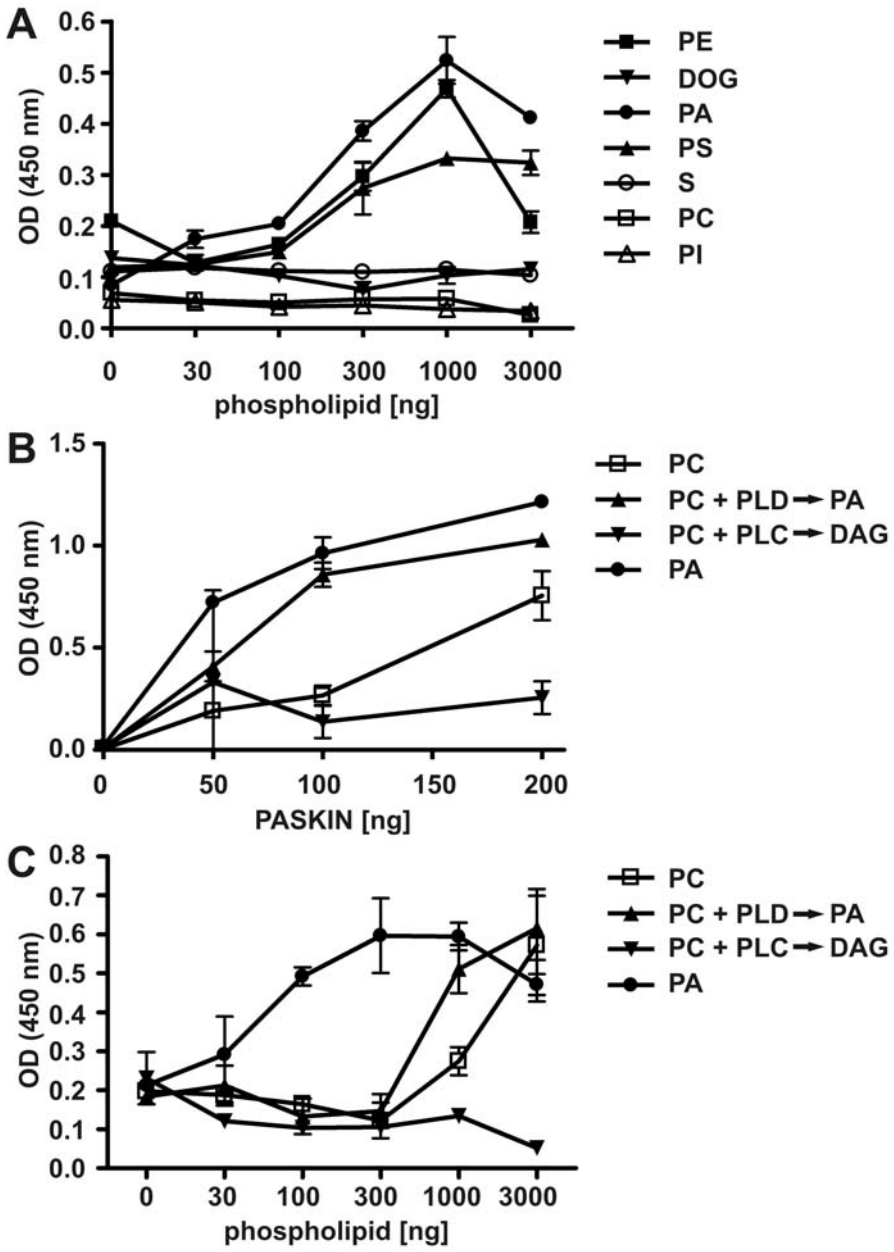

Figure 3: PASKIN-phospholipid interaction. A, 96-Well plates were coated with increasing amounts of the indicated (phospho-)lipids followed by incubation with purified PASKIN (100 ng). Following vigorous washing, PASKIN was detected with a monoclonal anti-PASKIN antibody followed by a secondary HRP-coupled antibody. B and C, PLD but not PLC converts PC from a low affinity to a high affinity PASKIN ligand. 96-Well plates were coated with constant (1 μg, B) or increasing (C) amounts of PC, followed by treatment with PLD or PLC. Binding of increasing (B) or constant (100 ng) (C) amounts of PASKIN was quantified as for A. B and C, Untreated wells coated with equal amounts of PC or PA served as controls. Shown are mean values ± SD of a representative experiment performed in triplicates.

Phospholipid stimulation of eEF1A1 phosphorylation by PASKIN

Because autophosphorylation of PASKIN has been suggested to increase its kinase activity [6], we next analyzed whether phospholipid-stimulated PASKIN autophosphorylation also leads to increased target protein phosphorylation. We previously demonstrated that eEF1A1 Thr432 is phosphorylated by PASKIN [14].

Unexpectedly, while PA induced PASKIN autophosphorylation it reproducibly inhibited eEF1A1 target protein phosphorylation (Fig. 4). This was not observed with PS and PE whose stimulatory activity on PASKIN autophosphorylation was slightly inhibited by the presence of eEF1A1.

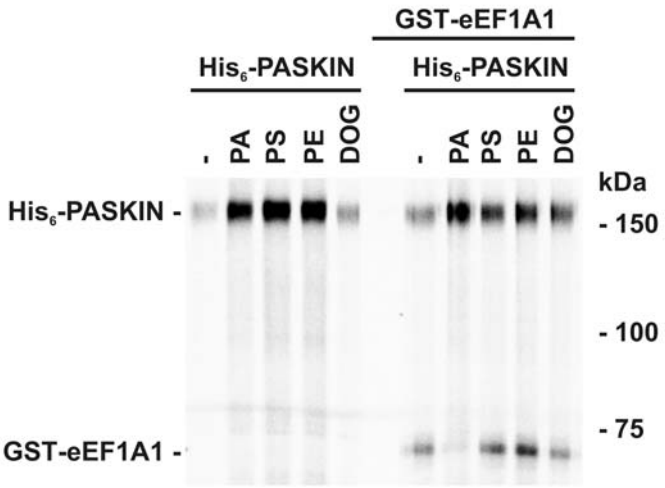

Figure 4: Effects of phosphoplipids on PASKIN-dependent eEF1A1 phosphorylation. PASKIN was stimulated with the indicated (phospho-)lipids in the absence (left panel) or presence (right panel) of recombinant GST-tagged eEF1A1. Following SDS-PAGE, phosphorylated proteins were detected by phosphorimaging.

DISCUSSION

Based on the known function of the PASKIN-related FixL oxygen sensor in bacteria, the role of the PASKIN orthologs in yeast, and the structural and functional studies of PASKIN in mammals, a ligand-dependent disinhibition of the kinase activity of PASKIN seems to be an attractive hypothesis. However, only synthetic but no endogenous PASKIN ligands have been identified so far and the functional implications of the binding of these ligands have not been assessed experimentally [7]. Having identified a target protein phosphorylated by both PASKIN and PKCδ [14], we wondered whether other similarities between PASKIN and PKCδ might exist. Indeed, phospholipids were identified as PASKIN ligands and efficient stimulators of PASKIN kinase activity. As expected from the different domain composition, our data revealed similarities but also clear differences between PASKIN and PKCδ stimulation by phospholipids. Apparently, the presence of a charged phosphate moiety is required for stimulation of PASKIN kinase activity, and PLD, but not PLC, can convert phospholipids from low into high-affinity PASKIN ligands. These data suggest that receptor-mediated intracellular signalling via PLD might regulate PASKIN function. However, up to date no extracellular ligands for cell surface receptors are known which would affect PASKIN function *in vivo*.

It has been suggested that glucose stimulates PASKIN expression and activity by an unknown mechanism. Activated PASKIN is thought to stimulate PDX-1 expression and nuclear import, leading to increased preproinsulin gene expression in pancreatic β-cells [10,11]. However, PASKIN phosphorylates PDX-1 Thr152 *in vitro* which apparently inhibits PDX-1 nuclear uptake [11]. Thus, there appears to be a contradiction between induced PDX-1 Thr152 phosphorylation by activated PASKIN and increased PASKIN-dependent nuclear PDX-1 function. Based on our findings, a model might be plausible which resolves this contradiction: glucose might stimulate an intracellular signalling pathway that activates PLD, leading to increased PA formation. PA in turn stimulates PASKIN autophosphorylation but inhibits PASKIN target phosphorylation like we found for eEF1A1. Thus, activated PASKIN might actually show decreased PDX-1 Thr-152 phosphorylation, allowing PDX-1 nuclear translocation. Indeed, it is known that glucose stimulates PLD activity and inhibition of PLD-dependent PA formation with butan-1-ol blocks PLD-dependent insulin secretion [18]. While we were unable to confirm a glucose-dependent increase in PASKIN mRNA levels [13], there might still be nutrient conditions that regulate PASKIN activity rather than PASKIN mRNA or protein levels. We hence aim to establish methods to measure changes in intracellular

PASKIN autophosphorylation and kinase activity following activation of the PLD signalling pathway.

Due to the concentration-dependent induction of PASKIN kinase activity, it has previously been suggested that PASKIN autophosphorylation within its kinase domain occurs *in trans* [6]. To directly assess how phospholipids lead to an induction of PASKIN autophosphorylation, we added PAS or KIN fragments of PASKIN to kinase assays containing the full-length PASKIN protein. However, neither the PAS nor the KIN fragments were phosphorylated by PASKIN. If anything, the presence of the KIN fragment inhibited PASKIN autophosphorylation. These data suggest that the entire protein is required to serve as a target for autophosphorylation, including the PAS domain that is known from several transcription factors to serve as protein-protein interaction domain and hence might also be required for PASKIN-PASKIN interaction to allow *trans*-autophosphorylation.

Currently, we do not know how phospholipids activate PASKIN autophosphorylation. PA and PS are bound by cysteine-rich motifs in PKC and Raf [15,19,20]. Binding of PA results in Raf-1 translocation to the inner surface of the plasma membrane, interaction with Ras and activation of MAP kinases [15,21,22]. However, whereas the kinase domain of PASKIN showed a high similarity to the kinase domains of PKC and Raf, PASKIN does not have any cysteine-rich sequences, suggesting a different PA binding site. Moreover, PASKIN immunofluorescence studies did not reveal any subcellular relocalization following treatment with PA, LPA or phorbol ester (data not shown).

With the identification of endogenous small molecule activators of PASKIN, we obtained the first hints on the upstream regulators of PASKIN activity and it will be a major future challenge to identify the signalling pathways and PASKIN-dependent downstream processes that are affected by these regulators.

ACKNOWLEDGEMENTS

The authors wish to thank J. Rutter for the generous gifts of plasmids, and D.P. Stiehl and G. Camenisch for helpful discussions.This work was supported by grants from the SNF (3100A0-104219) and the University Research Priority Program "Integrative Human Physiology".

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7. IDENTIFICATION OF NOVEL INTERACTION PARTNERS OF PASKIN

Identification of novel protein-protein interaction partners of PASKIN by yeast two-hybrid screening

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Key words: Energy homeostasis, Glycogen synthesis, insulin secretion, Nitrogen fixation, Protein phosphorylation, DNA-repair, Testis

ABSTRACT

PASKIN is a PAS domain AMP-related serine/threonine kinase highly expressed in the testis. Male PASKIN knock-out mice are normally fertile. Thus, the function of PASKIN in the testis probably becomes only apparent under stress conditions, like it is known from its yeast orthologs. Following disruption of normal testis homeostasis, PASKIN might sense the altered concentration of a (unknown) metabolic ligand, which triggers the downstream phosphorylation of (unknown) target proteins. We reasoned that the identification of such PASKIN interaction proteins will help to elucidate the function of PASKIN during spermatogenesis. Initially, a HeLa cell-derived cDNA library was screened with the *Matchmaker* yeast two-hybrid system using the PAS fragment of PASKIN as bait for protein-protein interaction. A translation factor was identified and its interaction with PASKIN could be confirmed by *in vitro* assays. Further screening of a human testis cDNA library using the kinase domain of PASKIN as bait revealed the interaction with proteins involved in DNA repair and signal transduction. Thus, yeast-two hybrid technology might provide useful informations on PASKIN function despite the lack of a haploid cell culture model which would be required to study the function of endogenous PASKIN *in vitro*.

INTRODUCTION

PAS (Per-Arnt-Sim) proteins are known from all three kingdoms of life and represent protein sensors involved in the perception of light intensity, oxygen partial pressure, redox potentials, voltage and certain ligands [1-3]. The mammalian PAS domain-containing proteins can also serve as heterodimerization interfaces of transcription factors involved in the xenobiotic response, adaptation to hypoxia and circadian rhythm generation [4-6].

By database searches using the PAS sequence as bait [7], we and other labs identified a novel mammalian PAS protein termed PASKIN [8] or PASK [9]. PASKIN contains two PAS domains showing a high structural similarity to the oxygen sensor protein FixL of *Rhizobium* species, and a serine/threonine kinase domain which is related to the AMP-kinases. As known from FixL, PASKIN represses its kinase activity in *cis*. Upon ligand-binding to the PAS domain of PASKIN, activation of the kinase domain takes place in *trans*, resulting in the autophosphorylation of the kinase domain [9]. Ligand binding as well as mutation of the PAS A domain results in the activation of the kinase domain. Synthetic ligands are identified and show a structural similarity to dioxin, known to bind to the PAS domain of the aryl hydrocarbon receptor [10]. However, an endogenous ligand for PASKIN activation has not been identified so far.

To elucidate the function of PASKIN, our lab generated PASKIN deficient knock-out mice. PASKIN is highly upregulated in the testis during the post-meiotic stage of spermatogenesis. PASKIN mRNA expression is remarkably higher in the testis compared to other tissues such as lung, liver and kidney. However, neither male fertility nor sperm production or motility was affected in PASKIN knock-out mice [11].

The first insights into target identification of PASKIN were described for the yeast PASKIN homologs PSK1 and PSK2. Under stress conditions, the psk1 psk2 double-mutant strain shows a decreased growth at elevated temperature (39°C) and galactose supply. PSK1 and PSK2 phosphorylate three translation factors (Caf20, Tif11 and Sro9) and two enzymes involved in glycogen and trehalose synthesis (Ugp1, Gsy2), coordinately controlling translation and carbohydrate flux [12]. Recently, it was shown that the mammalian glycogen synthase interacts with the midregion of human PASKIN and is phosphorylated at Ser-640 [13]. In a yeast two-hybrid screen for novel interacting proteins of PASKIN, we recently identified the multifunctional eukaryotic translation elongation factor eEF1A1 using a HeLa cDNA library. Interaction studies showed that the PAS A and the kinase domain of PASKIN interact with the C-terminus of eEF1A1. We could also demonstrate that eEF1A1 is mainly but not exclusively phosphorylated at Thr 431, probably leading to increased translation

elongation. Surprisingly, these two proteins showed a similar localization in the midpiece of the sperm tail, suggesting that phosphorylation of eEF1A1 by PASKIN might regulate translational elongation in sperm cells [14].

To verify the functional role of PASKIN in the testis, we repeated the yeast two-hybrid screening with a human testis cDNA library and the kinase domain of PASKIN as bait. One of the three identified putative interaction partners is the multifunctional protein Ku70 that is involved in a wide range of cellular processes such as apoptosis, DNA repair or transcription [15]. We could show that compared to the two other interactors, the inhibitor of activated STAT and the testis zinc finger protein, only Ku70 interacts with the kinase domain of PASKIN using *in vitro* GST pull-down assays. However, no phosphorylation by PASKIN was observed for Ku70.

MATERIALS AND METHODS

Yeast two-hybrid library and bait construction

A human testis-derived cDNA library was screened using the kinase domain of human PASKIN as bait according to the instructions provided by the manufacturer (Matchmaker; Clontech-Takara, Saint-Germain-en-Laye, France). Therefore, the full-length human PASKIN cDNA clone pDKFZp434O1522 [8] was digested with *Nco*I and *Sma*I and the cDNA fragment inserted into the *Nco*I-*Sma*I sites of the pLexA vector (Dualsystems, Zürich, Switzerland). Subsequently, the N-terminal part was deleted by *Sac*II-*Stu*I digestion and re-ligated to obtain pLexA-KIN.

Yeast two-hybrid screening

DSY-1 competent yeast cells were transfected with the bait vector pLexA-KIN. DSY/pLexA-KIN yeast cells were transfected with 50 µg of library cDNA and co-transformants were selected for resistance to medium lacking tryptophan, leucine and histidine. To eliminate background growth, 2.5 mM 3-amino-1,2,4-triazole (3-AT) a competitive inhibitor of the *HIS3* gene product, was used. Positive clones were further analyzed for growth on drop-out medium containing different 3-AT concentrations (0-50 mM), and for β-galactosidase activity to characterize the interaction intensity. When yeast cell passed this selection process, the AD-containing plasmid was isolated. Therefore, the yeast cells were centrifuged and lysed in 500 µl sorbitol buffer (3 M sorbitol, 0.5 mM EDTA, β-mecaptoethanol, 733 units/mg

lyticase) at 30°C for 30 min. The plasmid DNA was precipitated with 3 M potassium acetate and isopropanol as described by the manufacturer (Qiagen, Basel, Switzerland). Purified AD-containing plasmids were used for transformation of XL1-blue MRF' competent bacteria, which were selected on LB-ampicillin agar plates. Purified bacterial plasmid DNA was sequenced by Microsynth (Balgach, Switzerland). The sequences were analyzed and identified using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and protein (http://au.expasy.org/) search engines.

Plasmids

All cloning work for bacterial and insect cell expression was carried out using Gateway technology (Invitrogen, Basel, Switzerland). Human PASKIN cDNA (kindly provided by J. Rutter, Salt Lake City, UT, USA) was cloned into pENTR4 (Invitrogen) as described elsewhere [14]. To generate expression vectors for fusion proteins, ENTRy vectors were recombined *in vitro* with DESTination vectors using LR Clonase recombination enzyme mix (Invitrogen). pDEST15 and pDEST17 were used to generate bacterial expression vectors for glutatione-S-transferase (GST)- and His_6-tagged fusion proteins. pDEST10 was used to generate expression vectors for His_6-tagged PASKIN fusion protein in the baculovirus/Sf9 insect cell system (Invitrogen).

PASKIN expression and purification

His_6-PASKIN was purified from baculovirus-infected Sf9 insect cells using passive lysis buffer (Promega, Madison, WI) and Ni^{2+}-NTA agarose (Qiagen). His_6-PASKIN purity was checked by SDS-PAGE followed by coomassie or SYPRO ruby staining (Molecular Probes/Invitrogen), or by immunoblotting using monoclonal anti-PASKIN antibody mAb6 [14]. PASKIN purity was quantified by fluorescence imaging of the SYPRO ruby stained gels using a sensitive CCD camera (FluorChem8900, AlphaInnotech, Witec, Littau, Switzerland).

GST pull-down

Pull-down experiments were performed by mixing either purified proteins or 20 µl wheat germ IVTT (Promega, Madison, USA) reactions with 10 µg purified GST-tagged proteins or GST alone bound to glutathione sepharose beads. After 30 minutes incubation at room temperature in bead binding buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.01% NP40), beads were washed 3 times with washing buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.5% NP40), boiled in sample buffer (40 mM Tris-HCl pH 6.8, 1% SDS, 50 mM β-mercaptoethanol) for 5 minutes and the proteins separated by SDS-PAGE. Gels were stained with coomassie blue, dried and radioactively labelled proteins detected by phosphorimaging (Molecular Imager FX, BioRad, Reinach, Switzerland).

Kinase assays

100 ng His_6-PASKIN or 90 ng PKCδ (Invitrogen) were incubated with or without 2 µg bacterially expressed and purified GST-tagged proteins in kinase buffer (25 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) for 2 hours at 30°C in the presence of 3 µCi (γ-^{33}P)ATP (Hartmann Analytic, Braunschweig, Germany). Proteins were separated by SDS-PAGE and analyzed by phosphorimaging of the dried gels. Lipids were dissolved in $CHCl_3$, aliquoted in test tubes and the $CHCl_3$ evaporated under a stream of nitrogen. Lipids were then resuspended in kinase assay master mixes by thoroughly vortexing.

RESULTS

Identification of the novel PASKIN interactors Ku70, iSTAT and tZFP by yeast two-hybrid screening

A yeast two-hybrid screen with a total number of 7.9×10^4 colonies was performed with the kinase domain of PASKIN as bait and a human testis cDNA library. Three of 73 preys interacted with the pLexA-KIN fusion protein after retransformation and showed the correct open reading frame by sequencing. The other 70 initial clones were false-positives and could not be confirmed by retransformation assays or did not show the correct open reading frame. The following interacting proteins were identified: a subunit of the DNA-repair Ku complex named Ku70, the transcription factor testis zinc finger protein (tZFP) and the inhibitor of activated STAT (iSTAT). The initial growth phenotype of the prey Ku70 was observed up to

15 mM 3AT. After retransformation, the growth phenotype of Ku70 did not change (Fig.1 and Tab.1). In each case, the three clones were positive for signal β-galactosidase activity after retransformation (data not shown). The nucleotide sequence of Ku70 corresponds to the C-terminal end (nucleotides 891-2147) of Ku70 cDNA. The full length Ku70 has a predicted size of 70 kDa and protein domain searches revealed a protein-protein interaction site in the C-terminus of Ku70 (572-606 amino acids), termed SAP.

The initial growth phenotype of the two often potential interactors – tZFP and iSTAT – showed an enhanced growth up to 50 mM 3AT. After retransformation both putative interactors conferred yeast growth up to 15 mM 3AT (Fig.1 and Tab.1) sequencing revealed the correct open reading frame, matching with tZFP or iSTAT cDNA in the GenBank™ database. The nucleotide sequence of identified tZFP corresponds to nucleotide 211-1674. Two zinc finger motifs are located in the C-terminus of tZFP protein and represent potential DNA-binding sites, suggesting that tZFP functions as a transcriptional activator. The full-length of tZFP has a predicted molecular weight of 53 kDa.

iSTAT (predicted size of 63 kDa) contains the protein-protein interacting motif SAP as also known from Ku70. The functional role of iSTAT is the involvement in the JAK-STAT signal transduction pathway, regulating cellular processes like proliferation, differentiation and apoptosis [16].

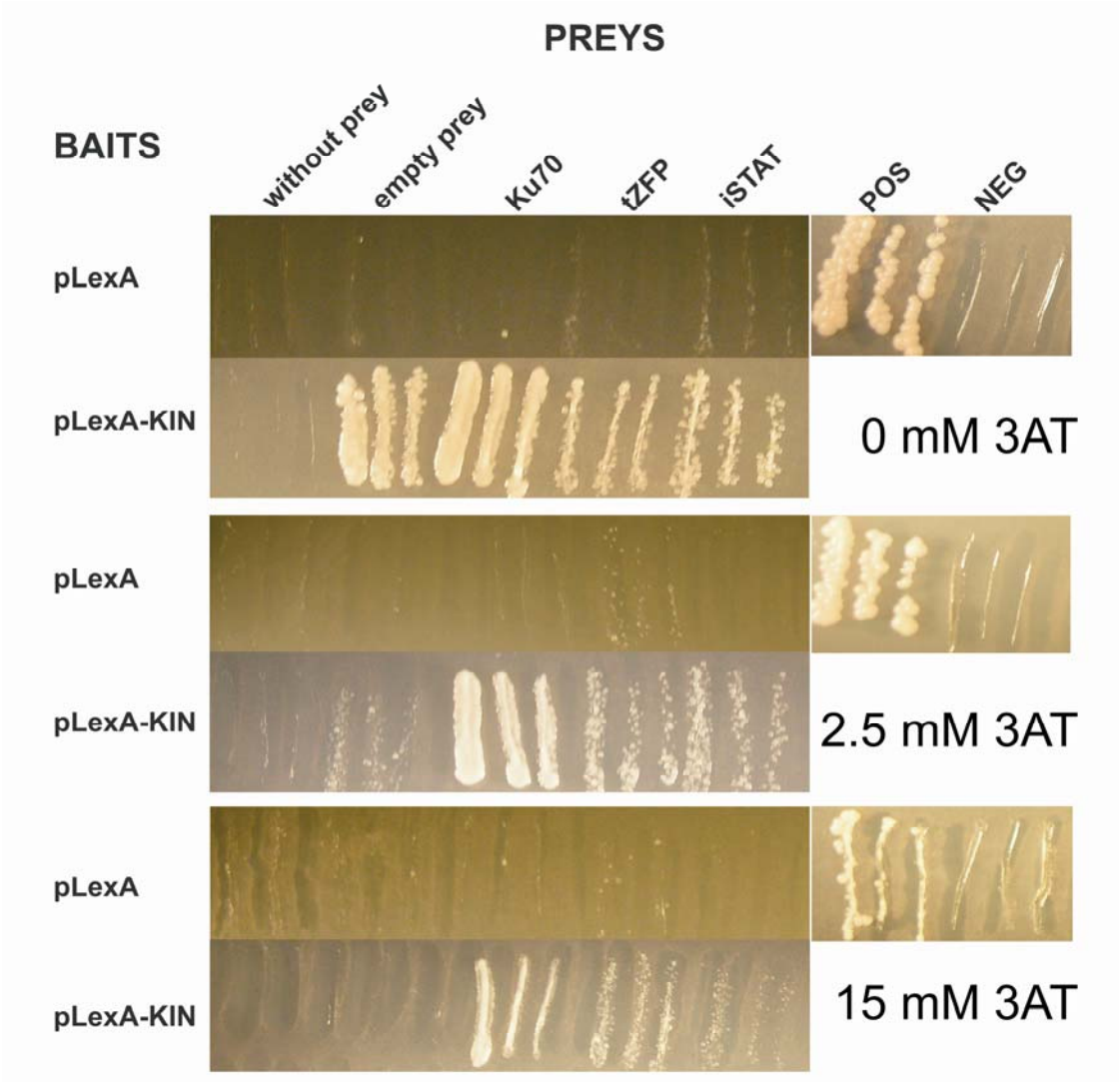

Figure 1: Interaction of the kinase domain of human PASKIN with Ku70 (274-609 aa), testis zinc finger protein tZFP (59-471 aa) and full-length inhibitor of activated STAT. After retransformation in yeast, positive clones were plated on triple nutrient selection (medium lacking tryptophan, leucine and histidine) and different 3AT concentrations, in comparison with positive (POS) and negative (NEG) control plasmids. POS: pAct2-LargeT and pLexA-p53, NEG: pAct2-LargeT and pLexA-LaminC. No interaction with any of the three putative interactors of PASKIN was observed with the empty bait vector pLexA.

Accession-number	PASKIN-Interactor	Protein size	Protein domain	Initial Phenotype		Phenotype Retransformation		Function
				3AT	β-Gal	3AT	β-Gal	
BC015190	Inhibitor of activated STAT	63 kDa	SAP	50 mM	++	15 mM	++	Inhibitor of STAT pathway
NM_001469	Thyroid autoantigen Ku70	70 kDa	SAP	15 mM	++	15 mM	++	DNA repair telomer maintenance V(D)J recombination transcriptional regulation
NM_014383	Testis zinc finger protein	53 kDa	Zinc finger motif	50 mM	++	15 mM	++	transcription factor

Table 1: Three potential proteins interact with the kinase domain of PASKIN in the yeast two-hybrid screen. After retransformation and sequence analysis, the Ku70 protein, a testis zinc finger protein and the inhibitor of activated STAT were identified as PASKIN interactors.

In vitro **interaction**

To determine whether Ku70, tZFP and iSTAT interacted directly with the kinase domain of PASKIN *in vitro*, proteins were bacterially expressed and purified as fusions to GST tag. The purified proteins were immobilized to glutathione-sepharose beads and incubated with the *in vitro* translated [^{35}S]-labeled kinase domain of PASKIN. After analyzing by phosphorimaging, the C-terminal part of Ku70 strongly interacts with the kinase domain of PASKIN and a weaker interaction was observed for the full-length Ku70 (Fig.2). The testis zinc finger protein and the iSTAT did not interact with PASKN *in vitro*. The eukaryotic translation elongation factor eEF1A1 served as positive control and the GST tag alone was used as negative control. In conclusion, we have shown that Ku70 is the only novel PASKIN interactor that could be confirmed by GST pull-down assays, showing an even stronger interaction with PASKIN *in vitro* than the positive control eEF1A1.

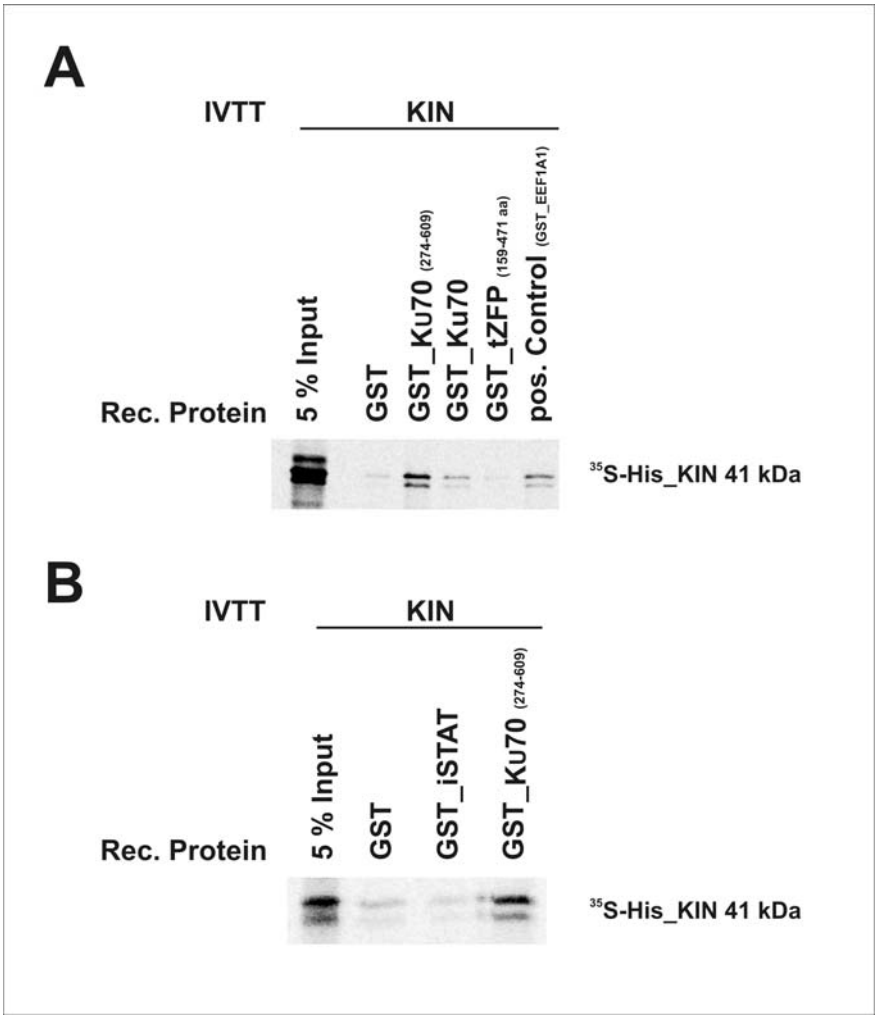

Figure 2: GST pull-down assay. IVTT (^{35}S)-labeled kinase domain of PASKIN was incubated with equivalent amounts of recombinant protein (Rec. Protein) GST alone or (**A**) GST fused to full-length eEF1A1, Ku70 and C-terminus of Ku70 and testis zinc finger protein (tZFP) (**B**) GST fused to full length inhibitor of activated STAT (iSTAT) and C-terminus Ku70. Input (5%) is indicated.

In vitro **target phosphorylation**

We previously reported that eEF1A1 is phosphorylated by PASKIN at Thr 431 [14]. In order to analyze whether Ku70 is also phosphorylated by PASKIN, kinase assays were performed. Therefore, PASKIN was purified from baculovirus-infected Sf9 cells and bacterially expressed and purified proteins fused to GST were used as targets. The autophosphorylation of PASKIN is stimulated by various phospholipids such as phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidylethanolamine (PE), but not diacylglycerol (used in form of dioctanoylglycerol, DOG) [17]. As shown in Fig. 3A the presence of phospholipids enhanced PASKIN autophosphorylation. However, Ku70 is not phosphorylated by PASKIN and the phospholipids PA, PS, PE or the second messenger molecule DOG did not stimulate PASKIN dependent Ku70 target phosphorylation. Compared to Ku70, the positive control eEF1A1 is phosphorylated by PASKIN. Interestingly, PA represses the phosphorylation of eEF1A1 by PASKIN.

It has been shown that the protein kinase PKCδ phosphorylates the eukaryotic EF1A1 at the same site like PASKIN [14,18]. Surprisingly, we could show that Ku70 is also phosphorylated by PKCδ and the target phosphorylation is enhanced in the presence of the classical PKC activators PS and DOG (Fig. 3B).

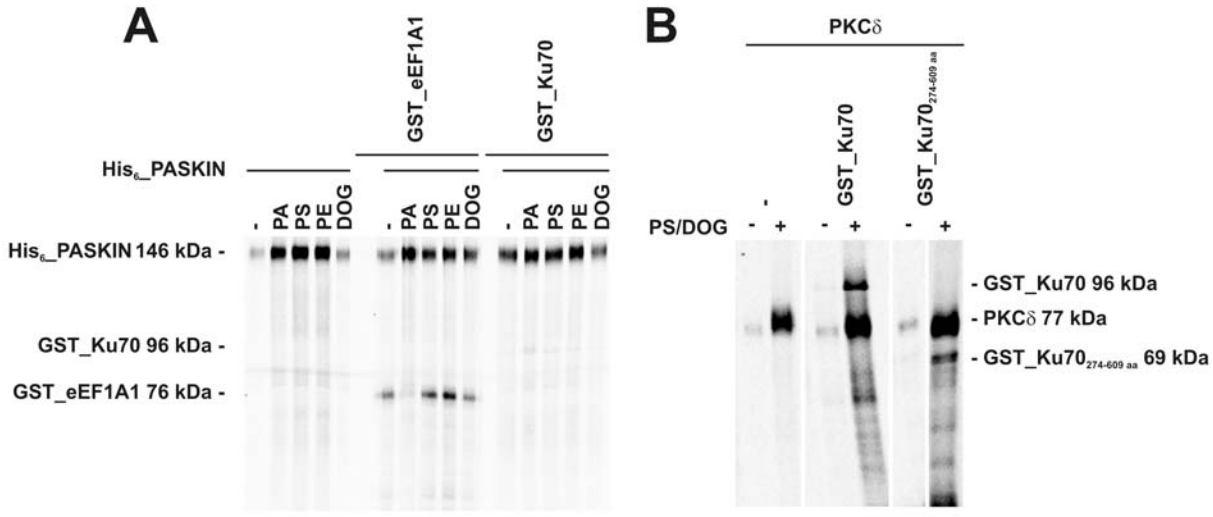

Figure 3: Phosphorylation assays. (A) PASKIN autophosphorylation and target phosphorylation with or without the phospholipids: phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylethanolamine (PE) and the second messenger molecule dioctanoylglycerol (DOG). The positive control eEF1A1 is phosphorylated by PASKIN and shows an inhibition of eEF1A1 phosphorylation in the presence of PA.

DISCUSSION

Our lab previously generated PASKIN knock-out mice which show a normal development, growth and reproduction. Surprisingly, PASKIN was found to be highly upregulated in the postmeiotic stage of spermatogenesis [11]. Because the absence of PASKIN did not influence male fertility, production or motility of sperms, the question of the functional role of PASKIN during the spermatogenesis arose. To get more insights into the function of PASKIN, a yeast two-hybrid screen with the kinase domain of PASKIN as bait and a human testis cDNA library was performed. From three putative interactors in yeast only one interactor could be confirmed *in vitro* by a GST pull-down assay. Following optimization of the experimental conditions, the yeast two-hybrid screening was repeated several times, but the average of the transformation efficiency of 6-8×10^3 clones/µg library could not be further enhanced to reach the predicted transformation efficiency of 10^4 to 10^5 clones/µg. The total number of 7.9×10^4 screened colonies reflects only a little part of the expected number of 3.5×10^6 independent clones (Clontech, manufacturer manual) of the human testis cDNA library.

The outcome of this yeast two-hybrid screening was the identification of the DNA-repair protein Ku70 interaction with PASKIN, which could be confirmed by GST pull-down assay *in vitro*. In contrast to eEF1A1, Ku70 is not phosphorylated by PASKIN *in vitro*, whether or not phospholipids were present. Ku70 is a multifunctional protein mainly involved in DNA repair as well as in many other cellular processes, including V(D)J recombination, telomere maintenance, transcription and apoptosis [15]. Recently, Ku70 and Ku80 were identified as interaction partners of the pancreatic duodenal homeobox-1 protein (PDX-1) in pancreatic β-cells [19]. The transcription factor PDX-1 is involved in glucose-stimulated insulin response and is phosphorylated *in vitro* by DNA-PK at threonine 11. Furthermore, Lebrun et al. demonstrated that radiation leads to a decrease of PDX-1 protein level and degradation of PDX-1 by proteasome. Decreased insulin promoter activity as well as the PDX-1-mediated target gene expression of *glut2* and glucokinase was also observed. Interestingly, promoter activity studies with the PDX-1 and preproinsulin genes showed that PASKIN increases promoter activity of both genes after elevated glucose concentrations *in vitro* [20]. Increasing glucose levels allegedly stimulate PASKIN activity in pancreatic β-cells and result in an upregulation of PASKIN mRNA expression and protein level [20]. However, our lab reported contrary data: PASKIN mRNA expression was not influenced after enhanced glucose stimulation in pancreatic β-cells. No differences in blood glucose levels and glucose tolerance were observed between wild-type and *Paskin* knock-out mice. The fact that PASKIN is highly expressed in the testis suggests that glucose stimulated insulin production in pancreatic β-cells

is independent of PASKIN [11,21]. However, PASKIN phosphorylates PDX-1 at Thr152 *in vitro* which probably inhibits the translocation of PDX-1 within the nucleus [22]. The observation that PASKIN as well as PDX-1 interact with Ku70, propose that PASKIN might be involved in phosphorylation of PDX-1 in a Ku70 dependent manner, thereby decreasing insulin promoter activity. The contrary data, whether PASKIN decrease or increase insulin promoter activity, propose a model which resolve this contradiction. Based on our finding that phosphatidic acid stimulates PASKIN autophosphorylation, but inhibits eEF1A1 target phosphorylation, these data suggests that PDX-1 phosphorylation is inhibited by PASKIN after activation of formed phosphatidic acid under high glucose conditions. Therefore, PDX-1 is translocated to the nucleoplasm, activating PDX-1-dependent gene expression [23-25]. It has been reported that other kinases such as PI 3-kinase are activated by elevated insulin concentration and stimulates nuclear uptake of PDX-1, resulting in further insulin secretion [26]. Low glucose level does not stimulate PASKIN autophosphorylation by phosphatidic acid. Hence, PASKIN might be able to phosphorylate PDX-1 at Thr152, resulting in inhibition of PDX-1 nuclear uptake and PDX-1-dependent gene expression. Therefore, Ku70 is able to interact with PDX-1 and DNA-PKcs-mediated phosphorylation of PDX-1 leads to degradation of PDX-1 by proteasome. PASKIN phosphorylation of PDX-1 at Thr152 might influence PDX-1 proteasomal degradation by stress-inducer such as low glucose concentrations. However, it would be interesting to investigate, whether overexpression of PASKIN influence PDX-1 proteasomal degradation in mouse embryonic fibroblasts (MEF's) derived from wildtype or Ku70 null mice after PA treatment or stress conditions such as low glucose concentrations.

In conclusion, Ku70 interacts with the kinase domain of PASKIN in yeast and *in vitro*, and is not phosphorylated by PASKIN. The lack of Ku70 phosphorylation suggests that PASKIN might phosphorylate Ku70-associated proteins such as PDX-1 leading to decreased promoter activity of insulin.

Additionally, we identified Ku70 as phosphorylation target of PKCδ *in vitro* which was unknown so far.

ACKNOWLEDGEMENTS

The authors thank J. Rutter for the generous gifts of plasmids and M. Hottiger for helpful discussions and support.

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8. CONCLUSIONS

PASKIN, a novel PAS AMP-kinase related serine/threonine kinase appears to be involved in a wide range of cellular processes. However, little is known about the functional role of PASKIN in the cell. To elucidate the function of PASKIN we searched for novel interaction partners of PASKIN to get more insights in the pathways PASKIN is involved. In a yeast two-hybrid screening, we identified the eukaryotic translation elongation factor eEF1A1. We could show that the C-terminus of eEF1A1 interacts with both the PAS A and the kinase domains of PASKIN in GST pull-down assay as well as in a mammalian two-hybrid system. Furthermore, we demonstrated PASKIN-dependent target phosphorylation of human eEF1A1 at Thr432. It has been reported that the multifunctional eEF1A1 is also phosphorylated by other kinases such as PKCδ, Rho-associated kinase and S6 kinase [1-3]. Interestingly, PKCδ-dependent phosphorylation site on eEF1A1 has been described for the corresponding mouse protein at Thr431 [1]. The physiological role of eEF1A1 phosphorylation by PASKIN is unknown so far, but suggests an involvement of PASKIN in translation elongation. Indeed, in a cell-free translation assay we could show that PASKIN increases protein translation. PASKIN knock-mice show normal development, growth and reproduction and revealed a high expression level of PASKIN in the testis during postmeiotic stage of the spermatogenesis. Surprisingly, PASKIN and eEF1A1 co-localized in the midpiece of human sperm tails using a newly generated monoclonal anti-PASKIN antibody. No corresponding cell culture model exists, hence we can only speculate that PASKIN might be involved in protein translation in human sperms. Apart from protein translation, it has been demonstrated that eEF1A1 seems to be involved in apoptosis [4-6]. Thus, we investigated whether PASKIN overexpression leads to apoptosis in testicular germ cell lines (NTERA, NCCIT, G-C1 and G-C2). None of the stressors like elevated temperature, hydrogen peroxides, serum free- or glucose-free medium induced PASKIN-dependent apoptosis (unpublished data). It will be interesting to investigate, whether stress-induced apoptosis differs in mouse embryonic fibroblasts (MEFs) derived from wildtype or PASKIN null mice. We demonstrated that endogenous PASKIN is localized in the cytoplasm as well as in the nucleus of HeLa cells and found PASKIN expression also in HEK293 cells. Using shRNA to stably downregulate PASKIN expression in these cell lines will help to elucidate the putative role of PASKIN during apoptosis for example by caspase-apoptosis-assays.

Because of the same eEF1A1 site is phosphorylated by PASKIN and PKCδ, we analyzed whether PASKIN might be activated by classical co-activators of PKCδ. Surprisingly, we

could show that phospholipids, but not the second messenger diacylglycerol, stimulate PASKIN autophosphorylation. Phosphatidic acid (PA), a charged phosphate moiety that is produced by PLD interacts with PASKIN in contrast to diacylglycerol generated by PLC. Interestingly, PA stimulates PASKIN autophosphorylation, but inhibits eEF1A1 target phosphorylation by PASKIN *in vitro*. These data have to be reproduced *in vivo* by a co-immunoprecipitation of PASKIN and eEF1A1 to check the phosphorylation status of PASKIN autophosphorylation and eEF1A1 target phosphorylation by anti-phosphothreonine antibody. Recently, it has been reported that glucose-stimulated insulin release leads to an increase in cellular PLD activity in pancreatic MIN6 cells [7]. The involvement of PASKIN in the regulation of PDX-1 is fortified by Rutter and colleagues. They conclude from their data that activated PASKIN enhances PDX-1 expression and translocation to the nucleus, leading to an increase in preproinsulin expression in pancreatic β-cells [8]. On the other site PDX-1 is known as phosphorylation target of PASKIN and is phosphorylated at Thr152 *in vitro*, leading to inhibition of PDX-1 nuclear translocation [9]. Based on our findings, a model is shown in figure 1 which might resolve this contradiction. Elevated glucose concentration might stimulate an intracellular signalling pathway that activates PLD resulting in increased PA formation. PA triggers PASKIN autophosphorylation, but inhibits PASKIN PDX-1 phosphorylation such as we observed for eEF1A1. It has been reported that PDX-1 is translocated by importin β [10] from nuclear periphery into the nucleoplasm [11,12] and activates transcription of genes such as preproinsulin [13], Glut2 [14] and glucokinase [15]. It has been reported that other kinases such as PI 3-kinase are activated by elevated insulin concentration and stimulates nuclear uptake of PDX-1, resulting in further insulin secretion [16]. Under stress conditions, such as low glucose, PASKIN might not be stimulated by PLD formed phosphatidic acid, leading to PDX-1 phosphorylation by PASKIN at Thr152. It has been reported that importin binds to the C-terminus of PDX-1 (146-206 aa), suggesting that PDX-1 phosphorylation at Thr152 inhibits the interaction of importin β and PDX-1 [10]. Thus, PASKIN phosphorylation inhibits the nuclear uptake of PDX-1 and insulin expression [9].

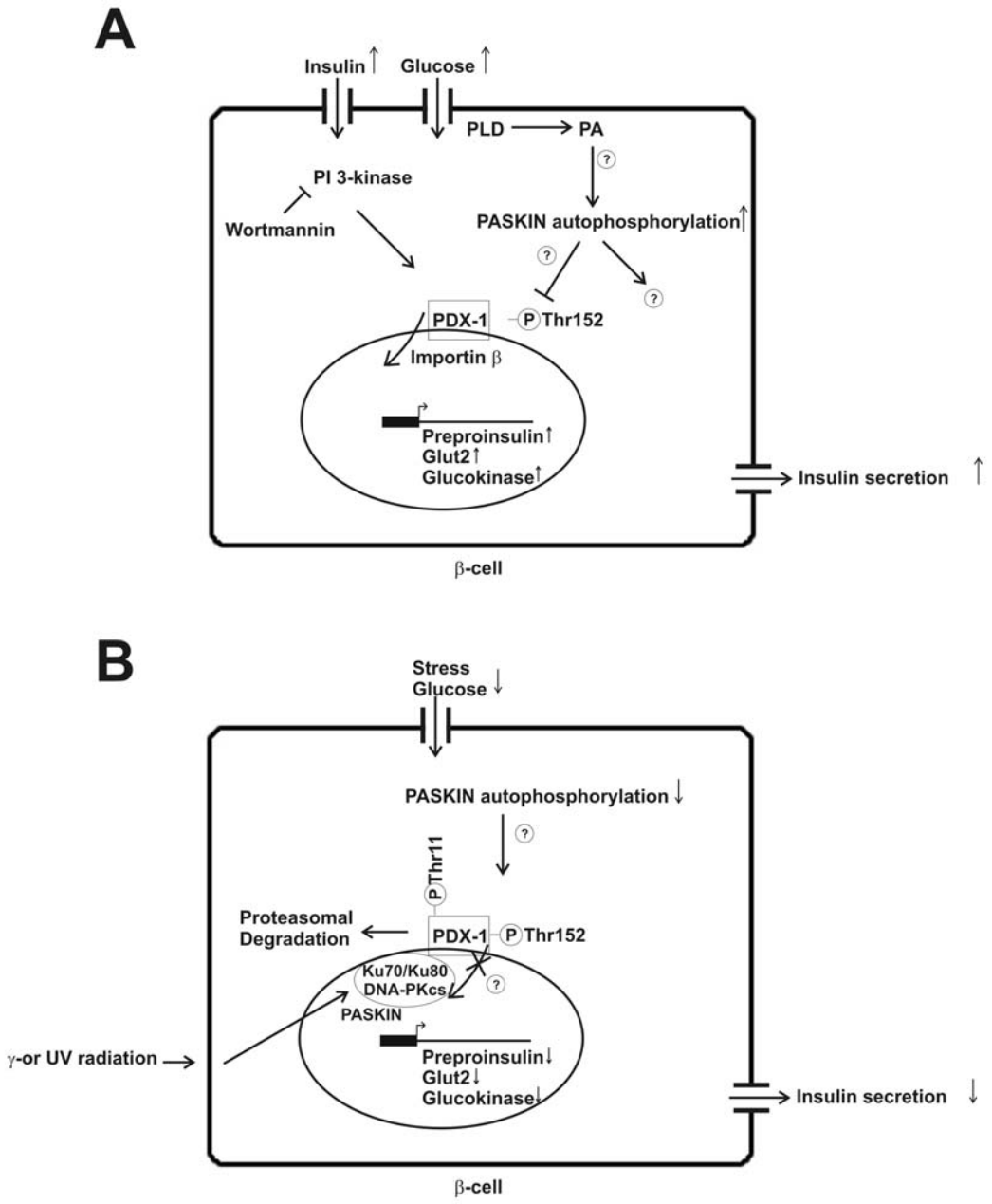

Figure 1: Hypothetical model for the involvement of PASKIN stimulation by phosphatidic acid in pancreatic β-cells. (A) Under high glucose concentrations PA is formed by PLD and stimulates PASKIN autophosphorylation, resulting in an inhibition of PDX-1 target phosphorylation by PASKIN on Thr152. Thus, PDX-1 is translocated by importin β from the nuclear periphery into the nucleoplasm. The transcriptional activation of PDX-1 target genes takes place and results in insulin secretion. By elevated insulin concentrations, other kinases such as PI 3-kinase are known to activate nuclear uptake of PDX-1, resulting in insulin secretion. (B) Under stress conditions such as low glucose concentrations PASKIN is not stimulated by PA and phosphorylates PDX-1 at Thr152. PASKIN target phosphorylation inhibits PDX-1 to translocate into the nucleoplasm. Stress-induced activation of the Ku70-Ku80-DNA-PKcs-complex e.g. by radiation leads to the phosphorylation of PDX-1 at Thr11, resulting in a proteasomal degradation of PDX-1. Low glucose as well as radiation show a decrease in PDX-1 target gene expression and low level of insulin secretion.

We identified the DNA-repair protein Ku70 as PASKIN interactor in a further yeast two-hybrid screening with a human testis cDNA library. This interaction could be confirmed in a GST pull-down assay. However, Ku70 was not phosphorylated by PASKIN *in vitro*. It has been reported that PDX-1 interacts with Ku70 and Ku80 in pancreatic β-cells and is phoshorylated at Thr11 *in vitro* by DNA-PKcs [17]. It has been shown that radiation leads to the proteasomal degradation of PDX-1 [17]. The observation that PASKIN as well as PDX-1 interact with Ku70, suggests a PASKIN-dependent phosphorylation of Ku70 interacting proteins such as PDX-1. In our lab, we could not confirm a glucose-dependent increase in PASKIN mRNA levels [18]. Therefore, nutrients might induce PASKIN activity rather than PASKIN mRNA or protein levels. It will be interesting to investigate whether PASKIN autophosphorylation is stimulated in a PLD-dependent manner in pancreatic β-cells as well as PDX-1 phosphorylation is inhibited by PASKIN after PA-treatment. Apart from insulin induced PLD activation, phosphatidic acid generated by PLD seems to be also involved in F-actin formation in sperm capacitation and acrosome reaction [19]. Immunofluorescence with an anti-PA antibody might reveal the same localization of PA in the midpiece of sperm cells such as we could demonstrate for PASKIN. In yeast is known that eEF1A1 influences actin interactions and bundling [20]. Therefore, we performed an actin bundling assay, but we could not show that PASKIN interacts with F-actin in this assay, suggesting that PASKIN does not regulate actin cytoskeleton organization (data not shown). It will also be interesting to repeat this F-actin bundling assay with PASKIN stimulated by phosphatidic acid.

By peptide and protein array screening, we identified further novel phosphorylation targets of PASKIN which are mainly involved in spermatogenesis, glycolysis and glycogen metabolism (data not shown). Among others, we could confirm the phosphorylation of mammalian glycogen synthase [21]. To confirm the phosphochip data and to reduce false positive substrates, we are planning an *in vitro* kinase phosphorylation assay on potential PASKIN target peptides.

In conclusion, we identified the eukaryotic translation elongation factor eEF1A1 as interaction partner and phosphorylation target of PASKIN. We could show that the PASKIN autophosphorylation inducer phosphatidic acid inhibts eEF1A1 target phosphorylation. The identification of phospholipids as endogenous ligands for PASKIN stimulation is the first hint on the upstream regulators of PASKIN activity. Finally, we need more insights into phospholipid stimulated PASKIN activation and the resulting target phosphorylation in pancreatic β-cells as well as in the testis to understand the functional role of PASKIN in mammals.

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9. OWN CONTRIBUTIONS

1.

Eckhardt K[1], **Tröger J** [1]**,** Reissmann J, Katschinski DM, Wagner KF, Stengel P, Paasch U, Hunziker P, Borter E, Barth S, Spielmann P, Stiehl DP, Camenisch G, and Wenger RH, 2007, Localization of the PAS domain kinase PASKIN and its novel target eukaryotic translation elongation factor eEF1A1 in male germ cells, **Cell Physiol Biochem** in press

[1]equal contribution

- PASKIN autophosphorylation and eEF1A1 target-phosphorylation (Fig. 6A and B)
- Sample preparation for Mass spectrometry (Fig. 6C)
- Linearization of expression vector, IVTT and immunoblotting for cell-free translation assay (Fig. 7A)
- Cellular translation assay (data not shown)

2.

Tröger J, Eckhardt K, Spielmann P, Schläfli P, Borter E, and Wenger RH, 2007, Phospholipid stimulation and downstream target identification of the PAS domain kinase PASKIN, submitted 2007

- Everything except figure 1 A-D

3.

Tröger J, Spielmann P, Katschinski DM, Camenisch G, and Wenger RH, 2007, Identification of novel protein-protein interaction partners of PASKIN using by yeast two-hybrid screening, *in preparation*

- Everything

10. CURRICULUM VITAE AND PUBLICATION LIST

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31.08. - 04.10.03	**European Congress of Protistology, San Benedetto del Tronto, Italy** **Oral presentation:** Morphology and Morphogenesis of *Didinium* cf. *nasutum*
13.12.05	**Seminar of Institute, Institute of Physiology, Zürich, Switzerland** **Oral presentation:** Identification of novel phosphorylation targets of human PASKIN
31.03 - 02.04.05	**Symposium: Stress signals and cellular responses – a heritage carried through evolution, Halle, Germany** **Poster:** Identification of novel protein-protein interaction partners of PASKIN
29./30.09.05	**ZIHP Symposium, Zürich, Switzerland** **Poster:** Inducible phosphorylation of translation factor eEF1A1 and DNA-repair protein Ku70 by PASKIN
06.10.05	**Annual Meeting of the Swiss Physiological Society, Bern, Switzerland** **Poster:** Inducible phosphorylation of translation factor eEF1A1 and DNA-repair protein Ku70 by PASKIN
26. - 29.03.06	**DPG, München, Germany** **Poster:** PASKIN: link between energy metabolism and protein translation in mammals?
04. - 06.12.06	**Targeting of the Kinome, FMI, Basel, Switzerland** **Poster:** PASKIN: link between energy metabolism and protein translation in mammals?

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([1]equal contribution)

Tröger J, Eckhardt K, Spielmann P, Schläfli P, Borter E and Wenger RH, **2007**, Phospholipid stimulation and downstream target identification of the PAS domain kinase PASKIN, submitted

Tröger J, Spielmann P, Katschinski DM, Camenisch G and Wenger RH, **2007**, Identification of novel protein-protein interaction partners of PASKIN using by yeast two-hybrid screening, *in preparation*

11. ACKNOWLEDGEMENTS

I would like to thank

my Doktorvater Dr. Roland Wenger for giving me the opportunity to work in his group and his guidance and continuous support.

Dr. Gieri Camenisch and Prof. Dr. Michael Hottiger for joining my Promotionskomitee and helpful hints.

Dr. Jared Rutter for providing me with useful plasmids and helpful informations.

my parents and my sister for their great support and open ear. A great thank for financing my computer to write my thesis.

especially Patrick for having always the time to discuss practical and theoretical problems, giving me comfort in stressful times and being a good friend.

Daniel for the support and having also time for helpful lab-discussion.

my lab friends Katrin, Patrick, Ela, Daniel, Reni for the excellent assistance and the fun in- and outside of the lab. I will miss them.

of course the other members of Rolis lab.

Ela, Patrick and Roli for reading the manuscript and critical comments.

the badminton-crew consisting of Katrin, Patrick and Daniel Steiner.

and all my friends, especially Guido, Laura, Sebastian, Fabio, Carsten, Gordon, Evi and Sabrina